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The effects of low-level microwaves on Na^+ and K^+ movements in human erythrocytes.

by

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submitted by Paul Douglas Fisher

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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When I was in the seventh grade I was faced with what I thought was an insurmountable task: finishing the seventh grade. My mother, in her wisdom, assured me that I would not only finish the seventh grade but many more.

to my mother

Lucille Fisher

and an equally wise mother-to-be, my wife,

Barbara Fisher

ABSTRACT

The influence of low-level 2450 MHz microwaves on Na^+ and K^+ movements across lipid bilayers and the erythrocyte membrane was studied. Suspensions of lipid vesicles or cells exposed to microwaves, absorbed power at a rate of 2-3 mW/ml. A number of discrete microwave effects on membrane Na^+ and K^+ transport were observed in the exposed systems. The effects appear to result from non-thermal or specific interactions of the microwaves with the affected transport systems ie: the effects are not a result of microwave heating.

Transport of Na^+ and K^+ via the ouabain sensitive $(Na^+ + K^+)$ATPase was inhibited by microwave irradiation. Independent observations that microwaves depressed, by 10-25%, ouabain sensitive $^{24}Na^+$ efflux, ouabain sensitive $^{42}K^+$ influx, and K-dependent ATPase activity led to this conclusion. Ouabain insensitive Na^+ efflux was enhanced by ~30%. The vehicle for the affected process remains unidentified. However, it may involve a ouabain insensitive Na^+/Na^+ exchange vector since the increased Na^+ efflux did not effect any net changes in cell Na^+ content. The passive fluxes of Na^+ and K^+ may also be increased in the presence of a microwave field. Support for the latter claim is not conclusive.

In the absence of a microwave effect on Na^+ leakage from sonicated egg PC and erythrocyte lipid vesicles, microwave

interactions with membrane proteins were probably responsible for the effects observed on intact membrane preparations. Resonant interaction of the microwaves with membrane proteins was invoked as the probable mechanism.

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There are many ways to describe what is involved in completing a Ph.D. However, my wife has informed me that the words I had intended to use would have tried the bravest of souls. The process is indeed taxing. That this work is now completed is itself a tribute to the patience and supervision of Dr. Mark J. Poznansky and Dr. W. A. Geoffrey Voss. They have my undying gratitude and respect.

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GLOSSARY OF ABBREVIATIONS

ADP - adenosine diphosphate

ATP - adenosine triphosphate

ATPase - adenosine triphosphatase

$(Na^{+}+K^{+})$ATPase - sodium and potassium activated ATPase

(Mg^{++})ATPase - magnesium activated ATPase

cal - calorie = 4.184J

db = $10 \log_{10}(P_1/P_2)$ where P_1 and P_2 are power levels

E - electric field vector; V/m

egg PC - egg phosphatidylcholine

eV - electron volt = 3.82×10^{-20} cal

H - magnetic field vector; A/m

hct - hematocrit

mW - milliWatt = 0.86 cal/h = 2.247×10^{19}eV/h = 10^{-3} J/s.

P - electromagnetic energy flux vector; W/m^2

P_a - time rate of energy absorbed (W)

P_f - forward power (W)

P_i - inorganic phosphate

P_{K^+} - permeability of potassium;cm/sec

P_{Na^+} - permeability of sodium; cm/sec

P_r - reflected power (W)

P_t - transmitted power (W)

Q_{10} - The temperature coefficient, the ratio of the higher to the lower rate at two temperatures 10 ^{o}C apart. Typically 2-3 for biochemical reactions

SAR - mass normalized specific absorption rate P_a/unit mass (W/kg)

VSWR - voltage standing wave ratio

μCi - microCurie, 3.7×10^4 becquerel (dps)

I - INTRODUCTION

1 : HISTORICAL NOTE

Prior to Hertz's demonstration of radiowaves in 1883 (108), studies of the biological effects of electricity focused on the physiological results of using a biological tissue as a conductive element. However, in 1893 D'Arsonval exposed mice and human beings to a 1-MHz electromagnetic field induced within a solenoid (18). He observed increases in metabolic rate and in internal body temperature of the subjects. Although the mechanism by which the heating effect was manifested was not yet known, D'Arsonval recognized the therapeutic potential of the heat for such ailments as arthritis (18, 109). However, D'Arsonval also acknowledged the possibility of athermal effects. He subjected diphtheria toxin to 200-KHz radiation for 15 min while maintaining the temperature of the sample at 18 ^{o}C. Animals injected with the irradiated toxin showed no ill effects while the controls all died within 26 hours (19). The attenuated toxicity was attributed to 'violent molecular vibrations' that somehow changed the structure of the toxin without changing its chemical composition. This was the first athermal biological effect attributed to the action of non-ionizing electromagnetic radiation.

The period after Marconi's demonstration, in 1899 (108), that

radiowaves could be an effective vehicle for wireless communication, saw notable improvements in the technology used in generating high frequency electromagnetic waves. Interest in the biological effects of such waves did not, however, resume until after World War One. In 1926, Schereschewsky (96) reported that exposure of mice to radiation at frequencies between 8 and 135 MHz caused lethal elevations in body temperature but that radiation in the 27 to 66-MHz range was especially effective. He suggested, pursuant to D'Arsonal's athermal hypothesis, that the increased effectiveness within that frequency range was due to non-thermal 'electromechanical vibrations within the cell'. This was the first report of an athermal frequency-specific biological effect of non-ionizing electromagnetic waves.

Unsuccessful attempts were made to resolve frequency-specific effects in Paramecia exposed to 10 to 75-MHz radiations (60), rats exposed to 9 to 12-MHz (62) waves, and mice exposed to 8 to 135 MHz waves (13). The frequency-specific effect reported by Schereschewsky (96) was suggested to be an artifact resulting from resonant coupling of the animal with his apparatus at the effective frequency (13). Interest in frequency-specific effects lapsed but was recently renewed with a suggestion by Fröhlich that membrane proteins may be specifically resonant in the GHz (10^9 Hz) range (35). Further, investigations of several groups have reported frequency-specific growth-rate effects in yeast-cell cultures exposed to low-level, 41.6 to 41.8-GHz radiation (20, 40). Others have observed similar effects on the growth of E. coli exposed to 64 to 76-GHz microwaves (11, 57).

D'Arsonval's work with diphtheria toxin was not followed up until 1930. The original experiments were repeated successfully with 158-MHz waves (80). More precise temperature controls were used, the toxin was repeatedly heated to 40 ^{o}C and cooled to 7 ^{o}C, but the attenuation of the toxicity by 158-MHz waves could not be matched by any other heating technique. In 1935, Szymanowski and Hicks (109), reported on the influence of 120 to 810-MHz waves on the lethality of botulinus, tetanus as well as diphtheria toxins. The potency of each of the toxins was attenuated by 2 to 6-h exposures (diphtheria toxin by 97%) and the degree of attenuation increased with the duration of exposure. No comparable effect was seen in control toxins although the absolute temperatures and mean rates of temperature change in exposed samples were exactly the same.

The medical implications of the work done on the effects of microwaves on bacterial toxins is at present unclear since no subsequent experiments have been done. No reports exist on whether the potency of these toxins is attenuated by microwaves after the animal is infected. The thrust of the reported observations of microwave effects on bacterial toxins is that, collectively, they indicate that biochemical changes as a result of athermal interactions are possible. Why this line of research was not persued at that time is not known.

Research on biological effects of microwaves again slowed down for the duration of the Second World War. As a result of the war, however, microwave technology and transmitting capacity developed to the extent that exposure of biological systems

became less of an academic curiosity and more of a public concern. Military and industrial personnel chronically exposed to microwave fields began experiencing a number of inexplicable subjective symptoms such as sore eyes and headaches (46). Two epidemiological studies on populations exposed to microwaves concluded that all of the symptoms displayed must have resulted from microwave heating (17, 6). Shortly thereafter, a maximal permissible exposure level (MPEL) for microwaves of 10 mW/cm^2 was recommended. There ensued a period of research, characterized by the Tri-Service research program (87) on the biological effects of microwave radiation, sponsored by the armed forces of the United States, in which it seemed the aim was to determine the validity of the 10 mW/cm^2 MPEL through basic research. For example, McAfee et al (78) observed that increased respiratory rates and blood pressure occurred in cats exposed to 1-MHz radiation, which correlated with fluctuations in subcutaneous temperature. They used this result to propose that undetected heating of subcutaneous nerve endings may be responsible for the apparently athermal neurologic effects of microwaves in chronically exposed persons (77). Simultaneously, however, a report appeared that resurrected the possibility of athermal biochemical effects of microwave exposure. Gunn et al (42) exposed the testes of rats to 24,000-MHz waves for 5 min and observed a decrease in testosterone regulated uptake of $^{65}Zn^{++}$ by the prostate. The effect was abolished by testosterone administration but no edema within the testes was observed to

account for the microwave induced drop in endogenous testosterone. Although microwave heating was noted, heating the testes to the same temperature and at the same mean rate by infrared waves did not affect prostatic uptake of $^{65}Zn^{++}$. More recent evidence of the influence of microwaves on testicular function also indicates an athermal effect on these organs. Increasing testicular temperature of rats to 60 ^{o}C by hot water or by infrared or 2450-MHz radiation arrested spermatogenesis (28). The hot-water and infrared treated groups regained spermatogenesis within 60 days, while the microwave treated group never recovered.

That microwave exposure can alter cellular differentiation athermally, as indicated by the above, has been shown in a number of embryonic systems. Pupae of Tenebrio molitor express teratogenic effects as a result of exposure to 9-Ghz (66, 68) or 10-GHz (12) radiation. No abnormalities were observed in conventionally heated temperature controls. Further, Fisher et al (29) and Van Ummerson (113) have shown changes in differentiation and rates of tissue growth in early chick embryos exposed to 2450-MHz waves; once again, an athermal influence appears to be necessary for some of the observed effects.

By the end of the 'Tri-Service' research period, an immense volume of Soviet and other Eastern European literature on the subject of the biological effects of microwaves became apparent. The common conclusion of these reports is that microwave radiation at levels greater than 0.01 mW/cm^2 caused pathological changes in chronically exposed personnel that were

not thermal in nature (46). The extent of their conviction is exemplified by the fact that the Soviet MPEL (39) was set at two to three orders of magnitude lower, depending on frequency, than the American MPEL, and that in the Soviet Union microwave radiation sickness is a recognized pathological condition with distinct stages requiring hospitalization (21, 46, 94). The criteria used to qualify the athermal effects reported in epidemiological studies reported by Soviet authors are subtle and almost exclusively subjective: insomnia, impotence, anxiety and amnesia, to name a few. The large volume of supporting literature (21, 23, 46, 61, 69, 70, 94, 101) alone lends credence to some of the clinical effects claimed to be a result of microwave exposure.

Bradycardia is one athermal effect of exposure to low-level microwave irradiation that is reported in the Soviet epidemiological literature (46, 94), and that is supported by more basic microwave experiments on heart rate in vivo and in vitro. Isolated turtle hearts exhibited bradycardia when exposed to 960-MHz radiation at an incident flux of 2 to 10 mW/cm^2 (71, 112). If microwaves had heated the heart of an ectotherm, one would have expected a tachycardic response. Indeed when power levels of the incident flux were increased to 16 to 40 mW/cm^2, heating was noted and heart rate increased. However, the microwave induced bradycardia was enhanced in the presence of the sympathetic blocker, propranalol hydrochloride, and the

induced tachycardia was enhanced when atropine was applied (112). When both atropine and the beta-blocker were applied together, microwaves had no effect on heart rate. These experiments were repeated on rat hearts irradiated *in situ* and identical results were obtained (82). Olsen *et al* hypothesized that the microwave radiation interacts differentially with the nerve remnants on the heart to produce the different effects. More specifically they suggest that microwaves affect the release of neurotransmitter or its interaction with receptors at the post synaptic membrane. A recent report, which supports this contention, indicates that 960-MHz microwaves at 1.5 mW/g decreases the apparent binding affinity between acetylcholine and acetylcholine receptors isolated from rat brain homogenates (72). The observed bradycardia (71) may therefore have been a result of depressed sympathetic activity rather than increased parasympathetic activity. Whichever is true, the reported influence of microwaves on heart rate is the first physiological effect attributed directly to the interaction of microwaves with an irritable membrane function.

The mechanism of the biological effects of microwaves must ultimately be resolved at the level of the enzyme systems involved in a physiological function that is sensitive to microwaves. To this end, studies of the behavior of proteins in electromagnetic fields began as early as 1941 (15, 16, 76). Soluble proteins in solution, treated as rigid dipolar spheres, are expected to rotate in response to an applied field alternating at frequencies of ~1 to 10-MHz. Examples such as

lysozyme and trypsin show relaxation frequencies of about 3-MHz (118). However, anomalous dielectric dispersions have been observed in solutions of small (<5 amino acids) oligopeptides at 115-MHz (76) 25-, 28- and 30-MHz (15) and in the range of 300 to 750-MHz (16). Although the relaxation of the molecule as a whole was not ruled out, it was aknowledged that dispersions may have occured as a result of the rotations of covalently bound subunits around the principle molecular axis, and of bound water (for an extensive review see ref. 121). That such resonances might be of biological significance was shown by Bach and colleagues (4). They examined the paper electrophoretic pattern of human gamma globulin exposed to 10-MHz and at frequency increments of 10-MHz up to 200-MHz. Although no effect was seen throughout most of that range, the single electrophoretic peak of the control sample was split distinctly in two when the sample was exposed to 30-, 60-, 140-, 180- and 200-MHz. All temperature controls, maintained at 25 to 30 ^{o}C, displayed a single electrophoretic peak. The effective frequencies were resolved more specifically in the 10 to 41-MHz range at increments of 0.02-MHz. For example, the electrophoretic peak of gamma globulin was split by 13.12-MHz radiation but not by 13.10- or 13.14-MHz. Further, the specific effective frequencies were shifted when the incubation temperature of the irradiated solution was increased to 37 ^{o}C. The changes in the electrophoretic pattern of irradiated gamma globulin provided an example of microwave-induced changes in proteins that were probably conformational in nature. Such frequency-specific and

temperature-dependent changes may occur in other protein systems as well. More recently, a similar effect has been observed in the liquid-gel-chromatography elution profiles of bovine serum albumin, ovalbumin and ribonuclease exposed to 10-MHz radiation (64). As a result of irradiation at an estimated field strength of 20.5 V/m, the proteins exhibited an increase in the apparent molecular radius of 0.27%, 7.6% and 22.6% respectively. Conformational changes caused by vibrational states induced by the applied field were considered responsible for changes in molecular radii that resulted in the shifts in the elution profiles.

The relaxation of protein segments in microwave fields of 1 to 500-MHz is a source of variability in the dielectric dispersion of biological tissues (99). Like the reactions of soluble proteins to alternating electric fields mentioned above, these reactions occur below the frequency range with which this thesis is concerned. However, Fröhlich has suggested that membrane proteins under the influence of a membrane electric field in the order of 10^4 to 10^5 V/cm become highly polarized and consequently have high dipole moments. This effect in combination with the fact that protein hydration causes vibrational damping in solution, led him to hypothesize stable vibrational (and probably rotational) frequencies in the order of 100,000-MHz for membrane proteins. Frequency-specific effects on bacterial (115) and yeast (20, 40) colony growth in the 40,000- to 80,000-MHz range tend to support Fröhlich's hypothesis.

The biologically interactive frequency range proposed by Fröhlich is too high to account for effects at 2450-MHz, the frequency used in this thesis. However, many membrane proteins are partly within the lipid bilayer and partly exposed to the aqueous environment on either or both sides of the bilayer. Further, the electric field of the membrane extends beyond the boundaries of the bilayer, the intensity of which ideally decreases proportional to the inverse of the distance from the bilayer surface. Portions of membrane proteins external to the bilayer are, therefore, variably polarized as a result of being subject to an electric field at a continuum of strengths and to motional damping proportional to the degree of hydration of that segment. Consequently, within the border portion of the membrane protein, one could envisage a full vibrational frequency spectrum between those inherent to soluble proteins and to those proposed as characteristic of inner bilayer protein segments ie.: 500 to 40,000-MHz.

Assuming vibrational states at 2450-MHz are possible in membrane proteins, there remains a question of the fate of energy absorbed from an externally applied field. Fröhlich's theory that vibrational interactions may result in a biological effect depends on the absorption of microwave energy at a specific site within a protein. It was previously assumed that all absorbed microwave energy would be evenly distributed among the

oscillatory modes of a molecule, ie: thermalized, but computer simulations have demonstrated that this need not be so (110). Instead of the absorbed energy being continuously redistributed, the energy of an induced vibrational mode may be coherently distributed, i.e: in an orderly, predetermined set of steps, within a macromolecule, as Fröhlich proposes.

Under normal circumstances an enzyme may absorb energy continuously from the environmental thermal energy pool via a particular vibration. This energy is distributed coherently through the enzyme to achieve a reaction. As the rate at which energy assimilated by that particular vibrational mode approaches saturation, ie: continuously surpassing the threshold, a small amount of energy absorbed at that point from some external source, like microwaves, might saturate that mode prematurely, thereby causing an increase in the reaction rate. The reaction rate might thereby be limited at some other step, at a lower temperature than expected, and be interpreted as a temperature specific increase in the reaction rate of the enzyme in response to an applied microwave field. Similarly, if the vibration excited by the external source is at a step in the middle of the usual sequence of coherent events, it could raise the energy of that step beyond threshold, causing a premature dissipation of stored energy and resulting in a decreased reaction rate. This proposed mechanism of interaction may account for the implied effects of microwaves on protein conformation and on the activity of a number of membrane proteins. The decrease in the apparent binding affinity of acetylcholine to the acetylcholine

receptor in brain homogenates exposed to 960-MHz fields (72) and the decrease in liver ATPase activity of rats exposed to 27-MHz fields (91) may be results of such specific interactions between microwaves and membrane proteins.

Some of the most persistantly reported and intensively debated microwave effects on membranes are those reportedly manifested as changes in cation flux, specifically, Ca^{++} flux in brain tissue and Na^{+} and K^{+} fluxes in erythrocytes. The effect of microwaves on Ca^{++} flux in chick brains has generated some controversy since the effect appears to have two frequency dependencies and a dependence on the rate of energy deposition in the tissue (122). Calcium flux was increased by exposure to 50-MHz, 147-MHz and 450-MHz microwaves but only if modulated at 16-MHz (9, 10, 58, 122). The most unusual specificity is that no microwave enhanced Ca^{++} flux occured if the rate of energy deposition exceeded ~1.4 mW/g (10). A possible explanation for the frequency dependence has been presented but the reason the effect is not present beyond a specific absorption rate, remains a puzzle. There are as many reports denying an effect on Na^{+} and K^{+} transport as there are supporting it. The Na^{+} and K^{+} transport systems of the human erythrocyte were chosen for study of the effects of microwave radiation on the function of the membrane protein. Accordingly, the value of the erythrocyte as an experimental system for the study of microwave bioeffects and the existing literature are discussed in the following section.

2 : MICROWAVE BIOEFFECTS AND THE ERYTHROCYTE

The erythrocyte or red blood cell is a relatively simple, biconcave disc shaped cell that is 7-8 μm in diameter and about 2 μm thick. It is the major cellular component of whole blood and exists primarily as a vehicle for hemoglobin transport and thus the transfer of oxygen between lungs and tissues. It also serves as one of the principal models of membrane systems in biophysical studies. The features that have made the erythrocyte one of the most extensively studied cells in the biophysics and biochemistry laboratory are:

1. its availability;

2. the ease with which it can be isolated as a homogeneous population in large quantities;

3. its relative simplicity: The human erythrocyte has no organelles, has relatively few different membrane or soluble proteins and has a well characterized and relatively consistent lipid composition; and

4. it is a consistently highly viable in-vitro system.

The lack of subcellular detail in the human erythrocyte limits the number of processes it exercises and, consequently, delimits the scope of biochemical reactions to microwaves that may be studied. On the other hand, the simplicity of the red blood cell makes it an ideal model for the in-vitro study of microwave effects on single-cell membranes. The few, but extensively studied, biochemical functions the erythrocyte does possess, may simplify attempts to elucidate mechanisms by which a particular microwave effect is manifested and to limit the possibility of effects occurring secondary to the primary

microwave interaction. Furthermore, the cells and vesicles in the suspensions used here exhibit Brownian Motion and thus have no fixed position. Consequently, each particle will absorb nearly the same amount of energy if all are restricted within the same small area of an electromagnetic field. The magnitude of any microwave effect relative to the energy absorbed can thus be more easily quantified. It is not surprising that the virtues of the erythrocyte have been previously recognized and used to investigate the biological effects of microwaves (7, 34, 44, 53, 54, 81, 86). The controversial nature of many of these reports is all the more reason to use red cells for this study.

One activity that the erythrocyte conveniently shares with other cell types is $(Na^+ + K^+)$ATPase-mediated Na^+ and K^+ transport across the cell membrane, producing opposite concentration gradients of Na^+ and K^+. In other tissues, such as intestinal epithelia, the transmembrane Na^+ concentration gradient supplies the motive force for the symport of some amino acids and sugars against their own concentration gradients (99). There is also some evidence that glycine and L-alanine transport in human red cells depend on Na^+ (119). In nerve cells the Na^+ and K^+ gradients established by the $(Na^+ + K^+)$ATPase and the differential permeabilities of each result in a net membrane potential that is essential for neuronal activity. One therefore has a convenient system, neatly packaged and accessible in the red blood cell, studies of which have implications for a number of other systems.

A disadvantage is the relatively low concentration of the

$(Na^+ + K^+)$ATPase in the erythrocyte membrane as compared with such tissues as kidney outer medulla and shark rectal gland (97). These systems, however, suffer the disadvantages alluded to above: They are not as easily available and are more difficult to isolate. The last objection can be avoided by extracting the protein and reconstituting it into an artificial bilayer; but detergent extraction of membrane proteins can compromise the function of reconstituted enzyme systems. There can be little question of the biological integrity of the erythrocyte as a model membrane system, a desirable feature for this study since mechanisms of microwave interaction are not fully understood, and artifacts of preparation would serve only to further complicate the investigation.

The conflicts and confusion surrounding the effect of microwaves on transmembrane cation movements is no less than the history of the field would suggest. In 1974 it was reported that rabbit red blood cells exposed at as little as 1 mW/cm^2 of 3000-MHz radiation showed increased K^+ and hemoglobin permeability and increased osmotic fragility (7). Attempts to reproduce these effects at 2450-MHz and 3000-MHz failed (44, 67, 86). The discrepancy was attributed to the lack of temperature control in the former investigation. However, Ismailov (54), and more recently, Olcerst et al (81), have conducted similar experiments in which mean temperature was very carefully controlled. Their results lend credence to the original claim that low-level microwaves increase passive cation permeability in erythrocytes, but neither observed increased leakage of

hemoglobin. Olcerst reported that increased passive Na^+ efflux from irradiated rabbit erythrocytes occurred only at temperatures correlated with discontinuities in the Arrhenius plot for passive Na^+ efflux. The temperature specificity may account for some of the previously conflicting reports (7, 44, 67, 86). Ismailov (53, 54), on the other hand, observed increased passive Na^+ and K^+ permeability in exposed human erythrocytes incubated at 37 ^oC. Ismailov also compared fluxes of Na^+ and K^+ from control and irradiated cells incubated with and without mono-iodoacetate [an ATPase inhibitor (51)]. He showed that, while microwaves increased the passive fluxes of Na^+ and K^+, irradiated cells also exhibited a decrease in the active fluxes of these cations. Perhaps, in those experiments in which no microwave effects on total Na^+ and K^+ fluxes were observed, the conditions were such that the opposing effects on active and passive fluxes were balanced, resulting in no net change in the exposed cells. This arguement was shown to be valid at least for the fluxes of K^+ in human erythrocytes exposed to low-level microwaves in the present study.

Little has been reported concerning the specific effects of microwaves on the activity of the $(Na^+ + K^+)$ATPase, the enzyme that performs active Na^+ and K^+ counter transport in erythrocytes and other cells. Microwaves of 1600 to 12,000-MHz

at 2 mW/cm^2 have been reported to be ineffective in changing $(Na^+ + K^+)$ATPase activity in guinea pig brain at 22 ^oC (107). However, during this experiment only 15-min exposures were used, and ATPase activity was measured after irradiation. Allis and Fromme report no effect of 2450-MHz microwaves on $(Na^+ + K^+)$ATPase in human erythrocytes at 37 ^oC (2). The validity of this report is suspect since the report makes no mention of ouabain or other specific inhibitors to resolve specifically the $(Na^+ + K^+)$ATPase activity from the large background of ouabain-insensitive ATPase activity in erythrocytes. Part of this study was done to fill the apparent void on the effects of microwaves on $(Na^+ + K^+)$ATPase activity and to correlate any changes to the effects on Na^+ and K^+ transport in human erythrocytes.

3:MICROWAVE EXPOSURE AND ABSORPTION

Microwaves occupy a portion of the electromagnetic spectrum, and are arbitrarily assigned the frequency range of 300 to 300,000-MHz (Figure I). The photon energies of microwaves is in the range of 10^{-7} to 10^{-3} eV, less than that of most biological processes: ie: 2.6×10^{-3} eV for Brownian motion at 30 ^oC to 10 eV for ionization (14). The mechanisms of microwave interaction with biological substrates are, therefore, qualitatively different from those of ionizing radiations. The photon energies of ionizing radiations (Figure 1) exceed biochemical bond energies and are, therefore, capable of splitting macromolecules. A quantum of microwave

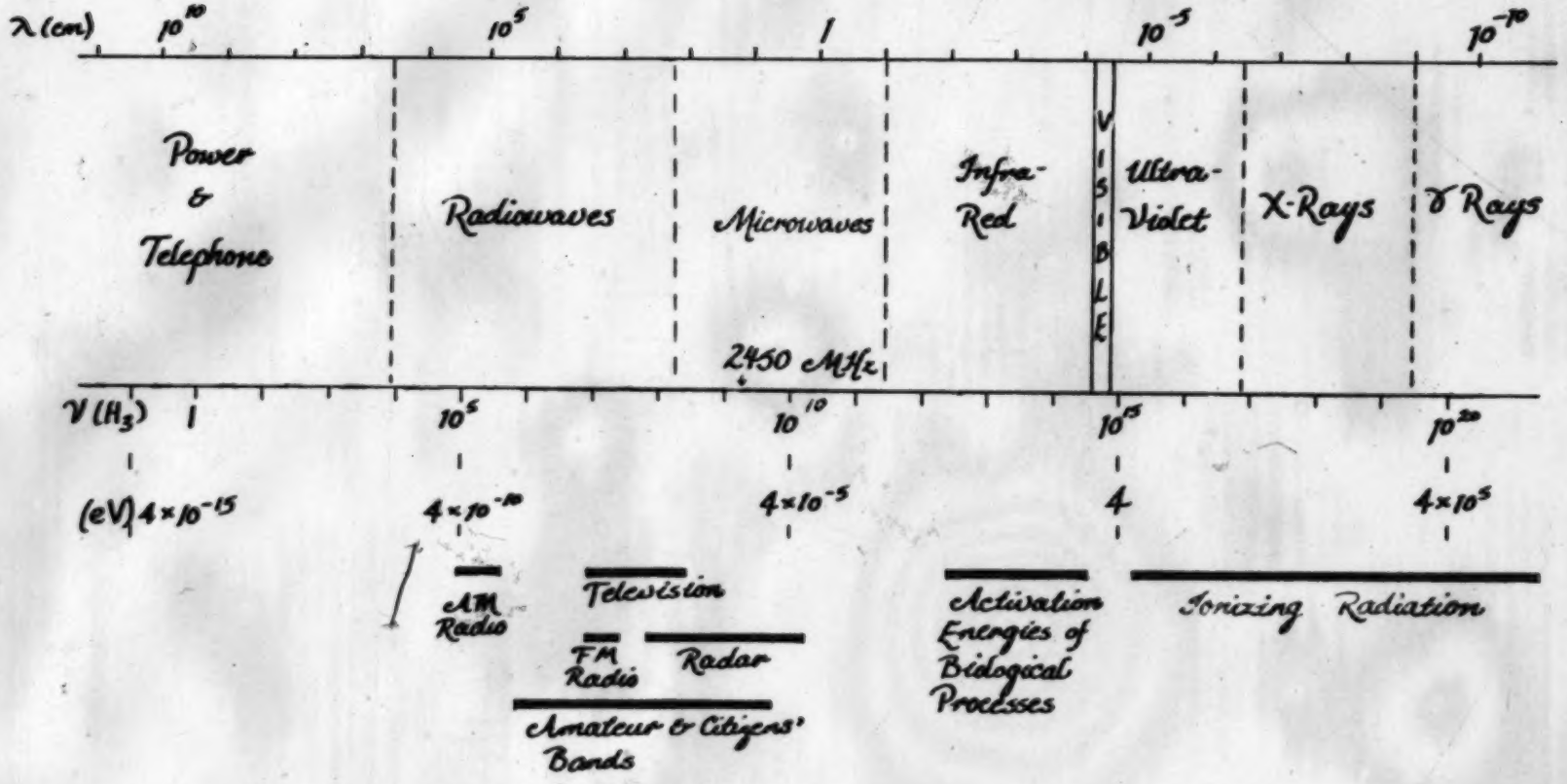


Figure I

The divisions of the electromagnetic spectrum designated by wavelength (λ), frequency (ν) and quantum energy (eV). Bands of some natural and man-made sources of radiation are also shown for comparison with the activation energies of most biological processes.

energy is in the same range as the vibrational and rotational energies within a molecule. Microwave energy may not, therefore, cause a primary chemical change but may alter vibrational and rotational motions within a molecule.

In order to begin to analyse a biological effect of microwaves one must first describe, at least subjectively, the conditions that dictate the manner in which microwaves are absorbed by a biological system. The mode and magnitude of energy absorption by a homogeneous body exposed to a microwave field is strictly determined by a number of physical parameters including:

1) the dimensions of the body, biological or otherwise, relative to the wavelength of the radiation

2) the orientation of the body relative to the direction of propagation; that is, the magnetic ($\bar{H}$) and electric ($\bar{E}$) field vectors

3) the electrical properties of the body, i.e: dielectric constant (ϵ) and conductivity (σ), and

4) The power levels of incident, transmitted and reflected energy with respect to that body, which ultimately determine the amount of energy absorbed.

Absorption of microwaves by electrically non-homogeneous bodies, such as the intact animal is more complex. The behavior of the electromagnetic field within and around each inhomogeneity must be considered. Most animals do not have the gross anatomical symmetry that would allow accurate calculation of field strength and distributions within the organism. Inhomogeneous masses within an organism may serve as dielectric lenses, or reflectors that focus energy, thereby causing electrical "hot spots" whch might in turn create a local increase in temperature or thermal

hot spot. Measuring energy losses within a field that contains a heterogeneous body is an estimate only of the average energy deposition to that body and provides no information about field pattern or local absorption. More direct approaches involve detailed thermal maps, or electric field probe measurements. There are problems inherent to the direct methods. Thermography (infra-red photography) does not have the penetration to resolve internal gradients in the intact organism. Implanted temperature measuring devices introduce artificial inhomogeneities or, if the device is an electrical conductor, may alter the field. Artifacts introduced by direct measurement may thus render extrapolation of data on distribution of energy absorption by an intact body questionable.

Our inability to describe accurately the pattern of microwave energy deposition in the intact organisms makes it difficult, if not impossible, to determine the precise site and mode of interaction responsible for a given biological effect. These problems may be circumvented by using electrically and biologically homogeneous systems isolated in vitro from an organism. However, an effect observed in vitro may or may not be manifested in the intact organism and vice versa. Homeostatic mechanisms may compensate in some way and thus mask a microwave-induced change. Considering, however, our limited understanding of biomolecular mechanisms attending microwave absorption it is probably more fruitful to try to elucidate biological effects in a sequence of systems of increasing complexity. Studying the biological effects of

microwave radiation in the reverse sequence, which has prevailed, in many cases, has confounded the issue of the mechanisms of microwave bioeffects.

The in vitro suspensions of lipid vesicles and human erythrocytes chosen for the present study are, on a macroscopic scale, electrically homogeneous within the microwave range. There are no electrical inhomogeneities within the cell suspension to alter the geometry of the field distribution within the suspension. On a microscopic scale, the suspensions studied here for biological effects of microwaves are not homogeneous. They are made up of particles (cells and vesicles) that in turn are inhomogeneous -- are composed of a suspension of proteinaceous particles bound by a membrane and an envelope of bound water. However, particles of this size influence the field distribution within the suspension only when the wavelength of the electromagnetic waves approaches the dimensions of these particles -- which, in this case, would be the far infrared range.

Absorption of microwave radiation is associated primarily with polarizing motions of small dipoles, such as water and ammonia. Most of the energy absorbed in this manner is quickly and evenly redistributed throughout the molecular motions of the system; i.e: is thermalised. The possibility of polarizing vibrations within biological macromolecules that may be integrated into the metabolic energy flow of the system, rather than being thermalised, provides a hypothetical basis for specific microwave effects. Regardless of the potential modes of molecular interaction with microwave radiation the magnitude of

the sum of all such interactions is determined by the frequency and the dielectric constant of a material at that specific frequency. The higher the dielectric constant of a material at a particular frequency, the more reactive the material is to electromagnetic radiation of that frequency. Dielectric relaxation techniques have clearly revealed that, at microwave frequencies, the principal biological substrate of interaction (and main determinant of dielectric constant) is water (99). Microwave energy absorbed by tissue water is undoubtedly thermalised, providing the intermediate step for thermal microwave effects. Interaction of microwaves with biological macromolecules is not as easily or clearly resolved by relaxation techniques due to the large number of different dipolar structures within and among biological macromolecules. That these interactions occur is supported by the observation that 30% of the total energy absorbed by muscle tissue exposed to 2500-MHz cannot be accounted for by water (33). The relaxation of biological dipoles has also been acknowledged as the source of deviations in the relaxation frequencies of tissue water compared with water alone (99). The probability of microwave interactions with biological macromolecules is the foundation on which existence of specific, non-thermal effects is based. However, resolution of non-thermal microwave bioeffects is often complicated because temperature sensitive, enzymatic processes occur in a predominantly aqueous environment. The sources and sinks of the thermal energy which invariably take place in a biological system exposed to microwaves, must be controlled or accounted for to accurately

assess the results of research aimed at resolving athermal effects.

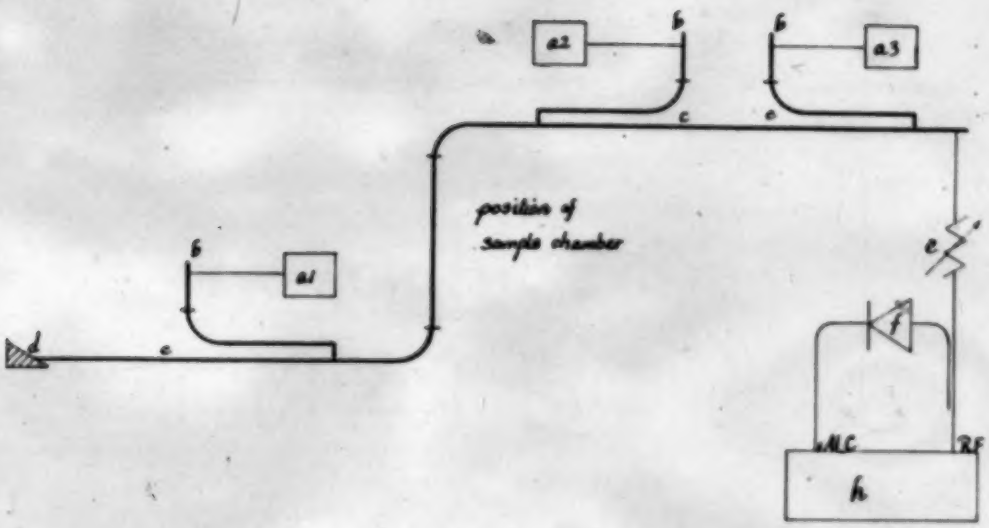
The processes studied in the subsequently described experiments all exhibit well-defined responses to temperature changes. In view of this, rather than perform simple temperature controls that may mask thermal effects, attempts were made to resolve athermal effects of microwaves on the continuous temperature responses of the systems that control Na^+ and K^+ fluxes across the red blood cell membrane. In fact, the effects of microwave exposure of red blood cells on some of the processes studied were clearly non-thermal. Whereas heat input would have caused an increase in the ouabain-sensitive flux rates and associated ATPase activity, microwaves caused the reverse effect.

II - EXPOSURE SYSTEM

1 : THE IRRADIATION SYSTEM

Biological exposure to microwaves in the environment is most likely to occur in a free field i.e.: one generated by some form of antenna such as that used for radar. Energy levels within a free space field vary as $1/r^2$ (in the far field only), where r is the distance between target and source, while the wavefront becomes progressively more planar. By virtue of the latter characteristic, this type of field is most convenient for the irradiation of large numbers of samples or animals but a relatively highly powered source is required to maintain a field of even moderate intensity in the far field of the antenna. A fundamental disadvantage of the free field exposure technique is that the only reliable measurement is the power density of the incident field. Absorption-rate measurements are thus averages and are prone to errors.

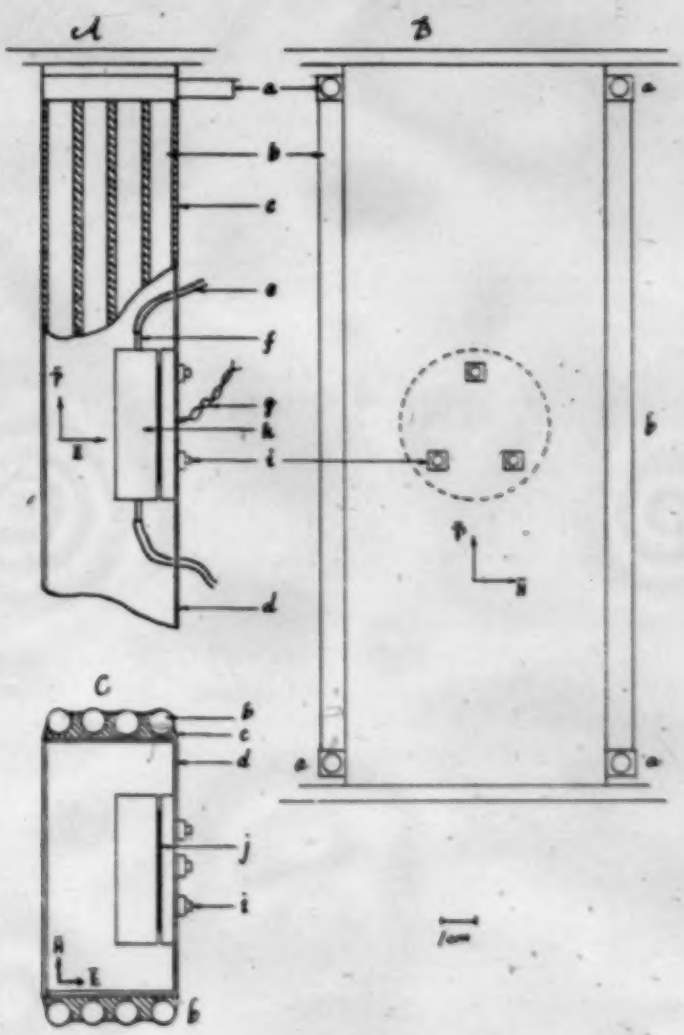
An in-waveguide exposure system was selected for these studies (Fig. II). In such a system there is, ideally, no attenuation of energy of the travelling wave through the length of the empty waveguide (in practice, ~0.036 db/m, for the waveguide used in this work). Accurate estimates of energy-absorption rates by samples during irradiation could, therefore, be made by measuring and comparing the forward (P_f), reflected (P_r), and



a - HP 431C power meters 1)-P_f 2)-P_r 3)-P_t
b - HP 471A thermistor mounts
c - Systron Donner DBL 675-10 directional couplers
d - HP 591A moving load
e - Narda Microline 791 FM attenuator
f - HP 420A crystal detector
g - PRD 544-10 directional couplers
h - HP 692D sweep oscillator or
American Microwave Inc. model 203 generator
RF - output
MC - auto leveling
(All coaxial cable (RG-8) to waveguide (WR-284) connections made with Douglas Microwave S BU-1015 adaptors).

Figure II

The waveguide irradiation system used to expose vesicle and erythrocyte suspensions to 2450 MHz microwaves.



a - access ports to heat exchange tubes
b - heat exchange tubes
c - metal epoxy
d - waveguide wall
e - sample access tubes
f - sample access ports
g - thermocouple
h - sample chamber
i - nylon bolts
j - neoprene O-rings

Figure III

One of the two waveguide sections modified to accommodate the sample chambers, in this case sample chamber #3, and temperature control system. One of these sections was incorporated into the waveguide exposure system and the other housed the control samples. The field pattern within the waveguide is indicated by the electric ($\vec{E}$) and magnetic ($\vec{H}$) field vectors and the electromagnetic energy flux vector ($\vec{P}$).

A - Cut-away side view with the sample chamber inside
B - Front-end view of waveguide section
C - End-on view of waveguide section

transmitted (P_t) energy with and without the samples in place. Unlike whole organisms, the geometry of an *in vitro* suspension relative to the field pattern does not change during irradiation. Consequently, microwave absorption by the samples is constant throughout periods of irradiation.

Sample chambers were constructed and mounted within waveguide sections (Figure III). The chambers were fastened to removable sections of waveguide wall. Tubes were passed through holes in the waveguide wall and connected to ports on the sample chambers, allowing access to the samples from outside the waveguide during irradiation. This feature permitted flow dialysis, sampling, and intermittent temperature measurement of the erythrocyte suspension during incubation. One of these waveguide sections, with a sample chamber, was part of the waveguide-exposure system (Figure II) the other stood, unperturbed, housing the control sample.

All but a small fraction of the typical suspension used in this work is water, a good substrate for microwave heating at 2450-MHz. The enzymatic processes studied here, specifically that of the $(Na^+ + K^+)$ATPase, show an acute dependence on temperature. To resolve the athermal effects of microwaves on this system it was necessary to consider the influence of heat from all sources, including microwave heating, on the activity of the enzyme. Consequently, temperature measurements were made as often and as carefully as possible. Furthermore, the objective of these experiments was to resolve microwave effects across a broad temperature range so incubation temperatures had to be precisely maintained. Temperature control of samples was achieved by

equipping the waveguide sections, containing the sample chambers, with heat-exchange tubes (Figure III) connected to a Polyscience Corp. Model-90 water bath with circulator. The selected temperature of the water bath was maintained ($\pm$0.2 ^{o}C) by feedback from a thermocouple in the water bath, via a Fluke model 2190A digital thermometer connected to the heater/refrigerator relay of the water bath.

The temperature of the exposed samples could not be monitored by microthermacouple during irradiation without perturbing the microwave field. However, a thermocouple was inserted into the control sample and the temperature thus recorded throughout the sample was identical to that monitored via the thermocouple embedded in the waveguide wall adjacent to the control sample chamber (Figure III). The temperature thus recorded remained constant (within 0.05 ^{o}C) throughout an experiment. The temperature of the waveguide wall adjacent to the exposure chamber was the same as that measured by inserting a thermocouple into the exposed sample immediately before or after irradiation or that monitored in the absence of microwaves. If there was microwave heating of the sample it was dissipated before it could be measured. Nevertheless, the temperature of each exposed sample was directly measured immediately after irradiation and this figure was used as the incubation temperature of that sample.

2:THE SAMPLE CHAMBERS

One of the most critical and hence controversial aspects of

experimental design in the study of the biological effects of microwave radiation is the containment of biological subjects or samples within the field. The difficulties arise in devising a system that is compatible with the biological assay techniques while minimizing the interactions of the container and measuring devices with the electromagnetic field. The latter, if significant, will ultimately affect the interaction of the microwaves with the system under study. Further, containment must be compatible with the protracted viability of biological processes. Several chambers were designed and built to satisfy these requirements and, as will be described, some of them were better suited than others.

The materials used in sample chamber construction were chosen for minimal absorption of microwave radiation relative to that of the sample and optimal compatibility with biological process. The former is determined by the dielectric properties of the materials. Table 1 lists these properties for the principle construction materials and for blood and salt solutions. Although the figures listed are for 3000-MHz radiation they differ only slightly from those at 2450-MHz. The real part of the dielectric constant (ε') reflects the ability of the material to contain an electric field and describes a strictly non-absorptive mode of microwave interaction. The loss factor or imaginary part of the dielectric constant (ε'') describes a material's ability to absorb electromagnetic radiation. The portion of energy flowing through a material that is absorbed by that material is proportional to the ratio of $\varepsilon''/\varepsilon'$, which is the dissipation

factor or loss tangent ($\tan\delta$). The $\tan\delta$ for the vesicle suspensions is between those values indicated for 100 and 300 mM NaCl (0.560 - 0.605) while that of blood is 3.363. The $\tan\delta$ values of the construction materials (2.0×10^{-4} - 4.8×10^{-2}) are much lower than those of the samples. Therefore, these materials absorbed much less of the microwave radiation, compared with that absorbed by the samples and provided the best available compromise with biological compatability and versatility.

Table 1

Real(ε') and Loss Factor (ε'') parts of the dielectic constants as well as the loss tangents ($\tan\delta$) of materials used in the experiments. Measurements are for 3000-MHz at 25-27 °C.

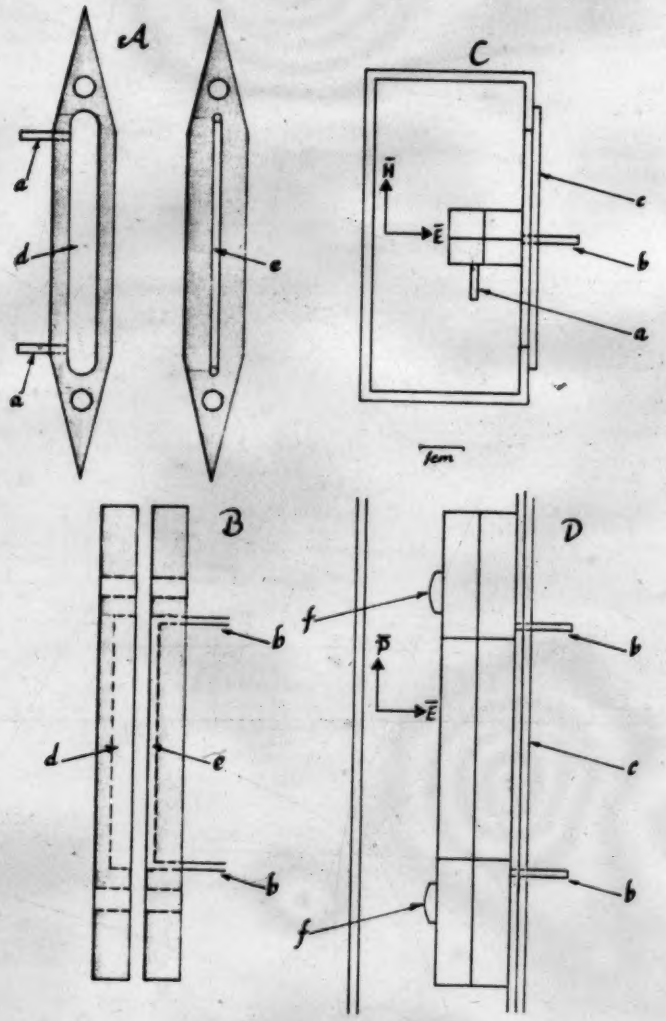
	ε'	ε''	$\tan\delta$	ref
human blood	17.1	57.5	3.363	111
300 mM NaCl	52	31.5	0.605	49
100 mM NaCl	54	30.3	0.560	49
Distilled water	80.5	24.9	0.310	49
Plexiglas	2.6	1.50×10^{-2}	5.7×10^{-3}	49
Neoprene	2.8	1.36×10^{-1}	4.8×10^{-2}	49
Parafilm	2.3	4.50×10^{-4}	2.0×10^{-4}	49
Glass	~5	$\sim 2.50 \times 10^{-1}$	$\sim 5.0 \times 10^{-3}$	49
Teflon	2.1	3.20×10^{-4}	1.5×10^{-4}	49
Polyethylene	2.2	6.80×10^{-4}	3×10^{-4}	49

Although the suspensions used were electrically homogenous, the whole sample presents an electrical inhomogeneity within the sample chamber and hence the waveguide system. The cross sectional area of surfaces perpendicular to the incident radiation were reduced, where possible, to minimize reflections,

standing waves and hot spots within the sample. These efforts also minimized standing waves within the waveguide, which affect power measurements due to loss of coupler directionality. The latter reduces potential errors in the SAR calculations vital to an accurate description of any microwave bioeffect.

The wavelength of the radiation within the sample, reduced (~15%) in proportion to the dielectric constant of the material through which it is passing, was not taken into consideration when determining the internal dimensions of the sample chambers. Due to the irregular geometry of the sample chambers the fields inside the sample are extremely complex. Accommodating the material wavelength in the sample chamber design would not have precluded standing waves within the samples. The complexity of the field within the sample chamber is of little biological consequence since the orientation and position of the particles (vesicles or cells) is always changing due to Brownian motion.

Sample chambers #1 (Figure IV) and #2 (Figure V), were used for the egg-PC vesicle and RBC-lipid-vesicle experiments respectively. The tapered ends of these chambers minimized reflections within the waveguide (Table 2). Chamber #2 (Figure V) is the simpler of the two, designed only to contain a vesicle suspension and to allow intermittant temperature measurement and sampling of the suspension. Chemical assays were performed after irradiation. Samples could be monitored by flow dialysis during irradiation by using sample chamber #1 (Figure IV). A dialysis membrane was placed between the halves of the chamber and was sealed at the edges with vacuum grease (Corning); the entire



a - sample access ports (nylon)
b - flow dialysis ports (nylon)
c - removable waveguide wall section (brass)
d - sample chamber
e - dialysate space
f - nylon screws

Figure IV

An expanded diagram of sample chamber #1 used in the irradiation suspensions of sonicated RBC lipid vesicles. Construction details are shown as well as the relative position in the waveguide relative to the $\bar{E}$ and $\bar{H}$ field components. Unless stated otherwise all materials were made of Plexiglas (acrylic).

A - FACE VIEWS OF BOTH HALVES
B - SIDE VIEW OF BOTH HALVES
C - END VIEW INSIDE WAVEGUIDE
D - SIDE VIEW INSIDE WAVEGUIDE

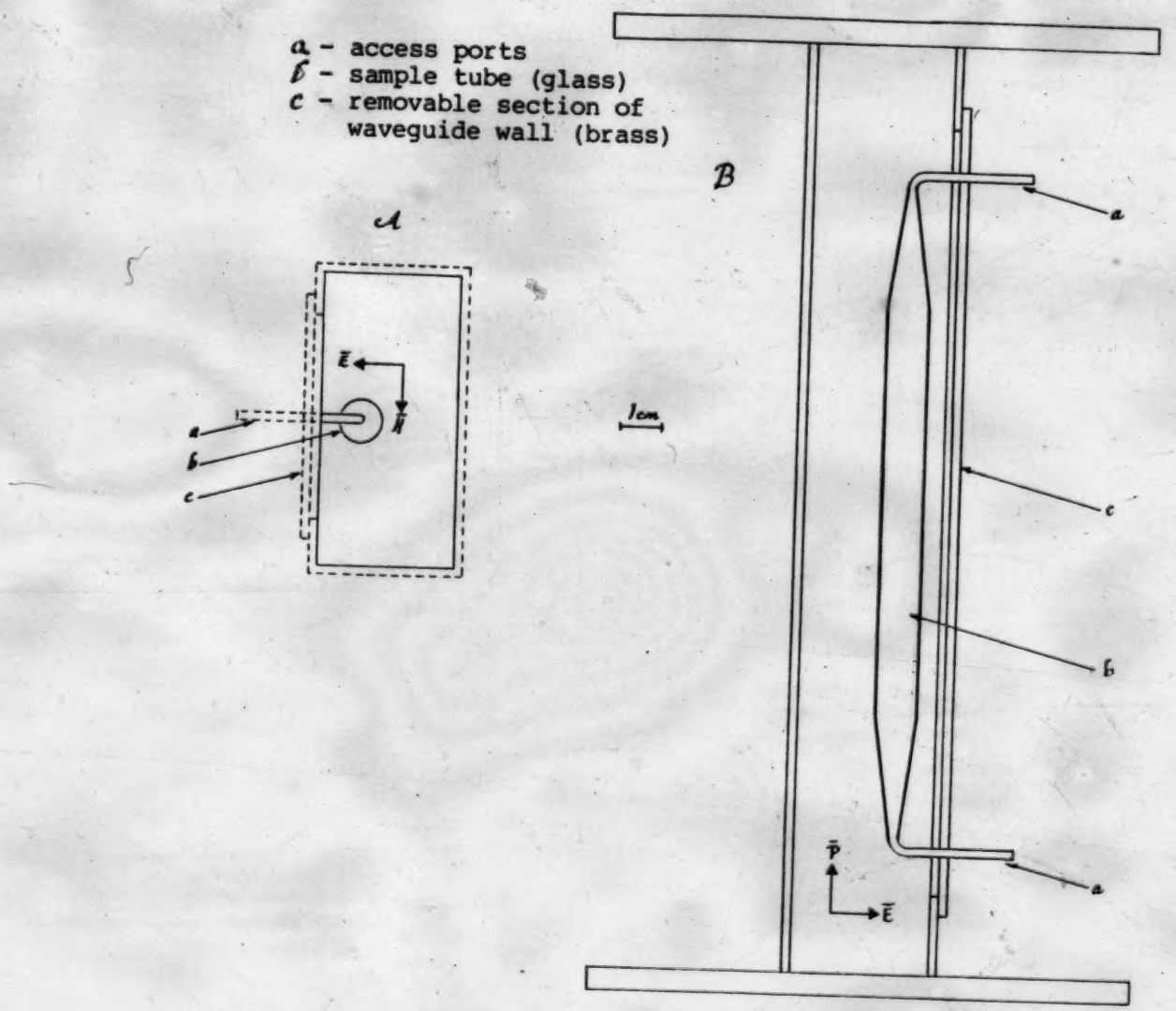


Figure V

Diagram of sample chamber #2 used in the irradiation of sonicated egg PC vesicles shown in position within the waveguide and with respect to the $\vec{E}$ and $\vec{H}$ field components.

A - view along waveguide axis B - cut away side view

assembly was bolted to a removable section of waveguide wall (Figure III). Cation leakage from the vesicles on one side of the membrane was thus continuously accumulated by the dialysate flowing through the trough on the other side. As with chamber #2, samples contained by chamber #1 were accessible during irradiation via the sample ports.

Table 2

The effects of the different sample chambers, with and without the samples, on the forward (Pf), reflected (Pr), transmitted (Pt) and absorbed (Pa) power levels, in mW, and on the voltage standing-wave ratio within the waveguide. VSWR's calculated from P_r according to VSWR Nomograph #1 in Ref. (93).

		P_f	P_r	P_t	P_a	VSWR
chamber #1	chamber empty	110	0	81	29	1.0a
	sample holder	110	0	81	29	1.0a
	+ sample	110	0	51	59	1.0a
chamber #2	chamber empty	110	0	82	28	1.0a
	sample holder	110	0	82	28	1.0a
	+ sample	110	1	78	31	1.2
chamber #3	chamber empty	110	0	82	28	1.0
	sample holder	110	10	71	29	1.8
	+ sample	110	17	61	32	2.2
chamber #4	chamber empty	110	0	82	28	1.0
	teflon holder	110	18	68	24	2.4
	holder & tubes	110	23	63	24	2.7
	+ sample	110	27	53	30	2.9

a - $P_r < 0.01$ mW, i.e. not detectable, therefore, $1.0 < \text{VSWR} \ll 1.1$

Sample chamber #3 described in Figure VI was the predecessor to chamber #2 and was used for Na+ efflux experiments. This

a - sample access ports
b - enoprene O-rings
c - sample space
d - dialysis membrane or parafilm
e - flow dialysis ports
f - Nylon bolts
g - removable waveguide section
h - dialysate space

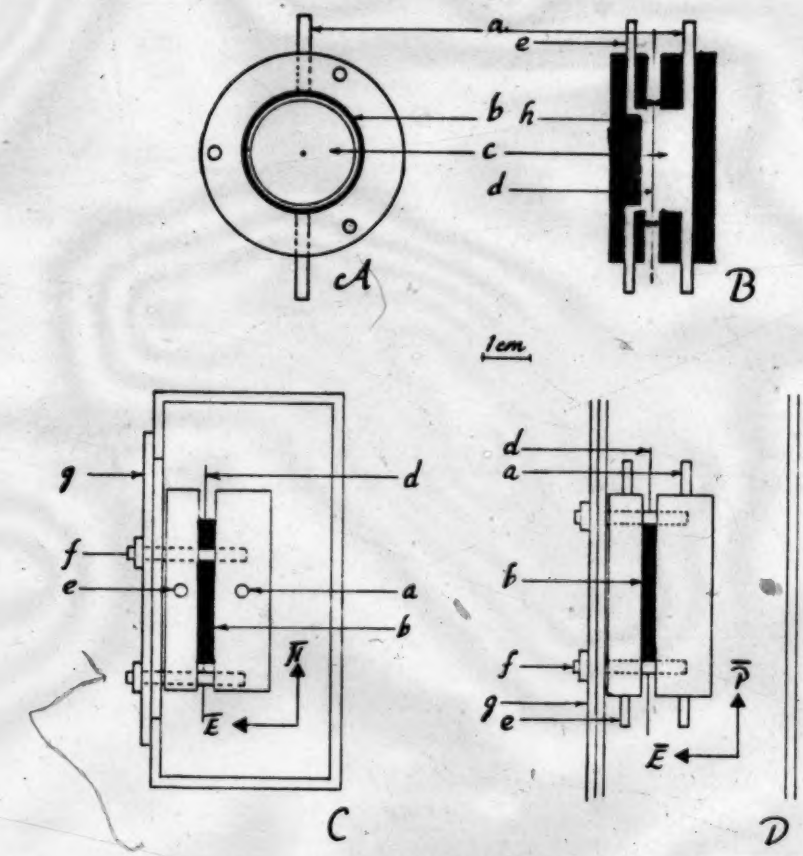


Figure VI

Expanded diagram of sample chamber #3 used in the irradiation of suspensions of red blood cells. Figures show internal construction details and the position of the chamber inside the waveguide with respect to the $\overline{E}$ and $\overline{H}$ field components. Materials are Plexiglas (acrylic) unless otherwise noted.

A - view along cylindrical axis
B - cut away side view
C - end view inside wave guide
D - side view inside

flow-dialysis unit differs from the other in shape and by incorporating neoprene O-rings in lieu of the vacuum grease. Although this chamber was less compatible with the irradiation system, as indicated by a VSWR of 1.8 (Table 2), it was used more extensively than chamber #2. The round dialysis unit was less prone to leaks and hence was a more equitable compromise in terms of biological compatability. This advantage, gained by incorporating O-rings, also reduced the risk of contamination of the sample with the vacuum grease needed to seal the halves of the tapered chamber. Chamber #3 was also used simply as a sample container by replacing the dialysis membrane with parafilm.

Table 3

The maximal possible VSWR contribution of the samples alone. Maximal errors in specific absorption rates determined from maximal conjugate mismatch losses (48).

chamber	maximum sample VSWR	sample volume (ml)	SARa mW/g	maximum error in SAR	
				%	mW/g
#1	1.00	4.5	6.5	4	0.26
#2	1.15	1	3.0	4	0.12
#3	1.22	1	3.0	14	0.42
#4	1.12	2	3.0	24	0.72

a - from P_f = 110 mW

The chamber least compatible with the irradiation system was

found to be #4 (Figure VII). The Teflon block used to hold two Eppindorf 1.5-ml centrifuge tubes was the primary contributor to the high waveguide VSWR (Table 2) and consequently caused the greatest SAR uncertainty (Table 3) as determined from the maximal conjugate mismatch losses (48). This two-tube arrangement, in sample chamber #4, however, allowed the irradiation of two samples simultaneously and the use of standard size centrifuge tubes facilitated the accurate processing of samples after irradiation. Because sample chamber #4 was so versatile it was used for the K^+ influx, ATPase activity and net Na^+ and K^+ flux experiments. In retrospect, however, a pair of chambers similar to #1 positioned symmetrically and in parallel within the waveguide would have been almost as versatile but more compatible with the waveguide system.

Power levels (P_f, P_r and P_t) measured within the waveguide system containing each of the sample chambers, and their components, are compared in Table 2 with the power levels measured in an empty waveguide. For the sake of comparison all measurements were made at P_f = 110 mW (albeit that this was not necessarily used in all experiments which are reported in the relevant sections). The VSWR was determined from the P_r, and the time rate of energy absorption (P_a) for each condition within the waveguide was calculated as:

$$P_a = P_f - (P_r + P_t)$$

Ideally there would be no attenuation within an empty

a - Teflon tube holder
b - 1.5 cc Eppendorf sample tubes (plastic)
c - nylon bolt
d - removeable waveguide wall section (brass)

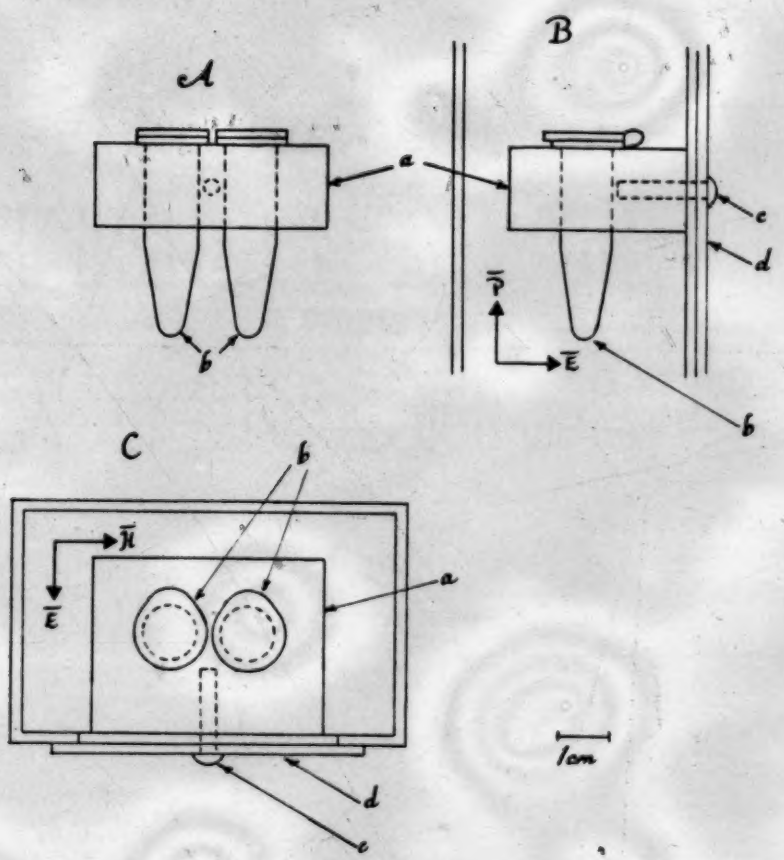


Figure VII

Expanded diagram of sample chamber #4 used in the irradiation of suspensions of red blood cells and red blood cell ghosts. Figures show construction details and orientation of the chambers within the waveguide and with respect to $\vec{E}$ and $\vec{H}$ field vectors.

A - FRONT VIEW
B - SIDE VIEW INSIDE WAVEGUIDE
C - END VIEW INSIDE WAVEGUIDE

waveguide but the losses within the system were far from ideal (Table 2). Power losses of this magnitude may have been caused by the large number of waveguide connections but more likely result from the two curved sections of waveguide incorporated into the system (Figure II) which are known to be particularly subject to loss. This attenuation within the empty waveguide did not affect the experiments but were accomodated in calculating the power absorption rates of the samples.

The empty sample chambers caused little further attenuation when introduced to the waveguide (Table 2), although chambers #3 and #4 contributed significantly to the VSWR within the waveguide. There was a further increase in the VSWR when the samples were put into the chambers (Table 2) but the maximal contributions to VSWR of the samples alone (if all standing waves are in phase), are calculated as:

$$(\text{VSWR})\ \text{sample} = \frac{(\text{VSWR})\ \text{total}}{(\text{VSWR})\ \text{empty chamber}}$$

were $\leq$ 1.22 for all chambers (Table3). However, mismatches due to the high VSWRs caused by chambers #3 and #4 resulted in large uncertainties, 14% and 24% respectively, in SAR measurements. The principle mismatch in experiments with chambers #1 and #2 was assumed to result from power meter coupling VSWRs, estimated at a maximum of 1.35 (48). Consequently SAR uncertainty in experiments with chambers #1 and #2 was only 4% (Table 3).

All the standing waves referred to thus far are within the waveguide; they do not reflect field conditions within the sample. The VSWRs shown in Tables 2 and 3 are only indicative of the suitability of the chamber and sample shapes to the irradiation conditions chosen for this study. These mismatches affected only one's ability to estimate accurately the rate of microwave absorption by our samples (Table 3) and do not directly affect the sample.

The above account of VSWR within the waveguide does not, however, negate the possibility of electrical or thermal hot spots within the sample. The conditions described simply indicate that if either exist, the field pattern responsible will be extremely complex. Data to be presented subsequently, however, indicate that the possibility of such a hot spot is small. The effects observed in red blood cells are not conventionally associated with heating nor could they be the result of thermal denaturation of any part of the erythrocyte. No thermal gradients were detected in any of the samples immediately after irradiation.

III LIPID VESICLE EXPERIMENTS

1 : INTRODUCTION AND PURPOSE

Cation flux across the erythrocyte membrane is mediated by a number of transport processes. Portions of the sodium and potassium fluxes, actively mediated by the $(Na^+ + K^+)$ATPase, can be eliminated in many cell systems, including erythrocytes, by as little as 5×10^{-7}M ouabain (25, 38, 59, 98). Small fluxes of these cations are, however, still evident in the presence of ouabain. In the absence of $(Na^+ + K^+)$ATPase, Na^+ and K^+ flux may occur by simple and/or facilitated diffusion. Diffusion may occur directly across the lipid bilayer or may be mediated by membrane proteins. An accurate description of the influence of microwaves on protein-mediated Na^+ and K^+ fluxes required an assessment of the effects of microwaves on simple cation diffusion across the exclusively lipid fraction of the erythrocyte membrane.

Sonicated vesicles of egg phosphatidylcholine (egg PC) and erythrocyte lipid extract (RBC-lipid) were used as models (5) of the erythrocyte lipid bilayer. Leakage of $^{24}Na^+$ from sham-irradiated vesicles and from those exposed to low-level 2450-MHz-CW field (expt V3) or a 3-Hz sweep in the 2350to 2550-MHz range (expt V1 and V2), was observed. Simple diffusion rates of Na^+ from control and irradiated vesicles were resolvedby use of the results of these experiments. Microwaves

at 2450-MHz, whether modulated or not, did not significantly influence the leakage of Na^+ either from sonicated egg-PC or RBC-lipid-vesicles.

2 : MATERIALS AND METHODS

2-1 : Erythrocyte Lipid Extract Preparation

Total lipids were extracted from one unit of packed human erythrocytes (provided by the University of Alberta Hospital Blood Bank) by use of a modification of a previously described technique (22). The cells were mixed with 5 vol CH_3OH and 5 vol $CHCl_3$ and were allowed to stand for 30 minutes with occasional stirring. The bulk of the proteinaceous precipitate was removed by filtration (Whatman #1). The filtrate was chilled to 4 ^oC and allowed to stand until the H_2O/CH_3OH and $CHCl_3$ layers had completely separated (~3 hours). The lipids were removed by rotoevaporation of the $CHCl_3$ layer and were resuspended in a small volume of $CHCl_3$ to which was added 10 vol $CHCl_3$, 1 vol CH_3OH and 0.75 vol, 0.1 M KCl. This mixture was vigorously shaken in a separatory funnel and allowed to stand at 4 ^oC until the H_2O/CH_3OH and $CHCl_3$ layers had completely separated (~30 minutes). After rotoevaporation of the $CHCl_3$ layer, the lipids were resuspended in benzene (20.66 mg lipid/mg benzene) and stored frozen (-20 ^oC) until required.

2-2 : Experimental Procedure

For each of experiments V1 through V5, egg-PC (Makor Chemicals, Israel) or RBC-lipids were suspended in the chilled

buffer solutions described in Table 4. The suspensions were then sonicated at 4 °C under nitrogen according to the specifications listed in Table 5. Transbilayer cation diffusion may be affected by lipid oxidation (5, 85). Measures were taken, while preparing lipid vesicles, to minimize lipid oxidation: elimination of unnecessary air contact and flooding air-spaces with nitrogen (52, 65, 83).

Table 4

The chemical makeup of the lipid suspensions that were sonicated to produce lipid vesicles in each of experiments V1 through V5. All buffers were adjusted to pH=7.4 with HCl.

exp #	lipid type	buffer constituents (mM)						
		NaCl	KCl	Tris/HCl	$MgCl_2$	Cysteine	Na_2HPO_4	Dextrose
V1	egg-PC	130	20	10	5	0	0	0
V2	egg-PC	130	20	10	5	0	0	0
V3	egg-PC	145	4	0	1	0	2.5	11
V4	RBC	100	10	30	5	1	0	0
V5	RBC	100	10	30	5	1	0	0

Table 5

The specifications for the sonication procedures used in experiments V1 through V5.

exp #	suspension volume (ml)	suspension lipid conc. (mg/ml)	ultrasonication(a) type	ultrasonication(a) duration (min)	ultrasonication(a) power (watts)	vesicles trapped volume (ml/gm)	vesicles diameter (nm)
V1	2.0	25	bath	90	80	1.0	21
V2	2.0	25	bath	90	80	1.0	21
V3	2.0	25	probe	45	50	1.0	21
V4	1.5	14	probe	45	50	2.2	60
V5	1.5	14	probe	45	50	2.2	60

a - During bath sonication (Labratory Supplies Co. Hickerville, N.Y. Model G112SP1G) energy was delivered to the whole bath including the sample. Energy delivered to the sample is, thus, much less than 80 watts. During probe sonication (Heat Systems-Ultrasonics Inc. Plainview, N.Y. Sonifier Cell Disruptor W185) the energy stated is that delivered to the microprobe in the sample.

In each buffer a portion of the Na^+ was as $^{24}NaCl$ (20 μCi/mg for experiments V1 and V2) or $^{24}NaOH$ (14 μCi/mg for experiments V3, V4 and V5) provided by the University of Alberta Slowpoke Facility. Some of the $^{24}Na^+$ (2-3%) was, therefore, trapped inside the vesicles when formed. Extravesicular $^{24}Na^+$ was removed by gel chromatrography (Table 6). The same buffer used in sonication, excluding the $^{24}Na^+$, was used as the eluent. This procedure also allowed the separation of unwanted multilamellar vesicles and removed any titanium particles that may have dispersed during probe sonication. Typical resolution

of the vesicles on a Sepharose 4B column is shown in Figure VIII. The aliquots of eluent, collected in 3 or 5 minute increments, coincident with the vesicle peak (Figure VIII) were pooled and used in this form for the experiments.

Table 6

Specifications of the pre-exposure chromatographic procedures used to separate unilamellar vesicles from multilamellar vesicles and extravesicular $^{24}Na^{+}$.

exp #	type	flow rate (ml/min)	void volume (ml)	column dimensions (cm)
V1	Sephadex G-50	6.5	39	25 x 2.5
V2	Sephadex G-50	6.5	39	25 x 2.5
V3	Sepharose 4B	1.0	30	25 x 2.5
V4	Sepharose 4B	0.8	22	25 x 2.0
V5	Sepharose 4B	0.8	22	25 x 2.0

The experiments were carried out under the criteria specified in Table 7. At the end of each experiment the control and exposed samples of egg-PC vesicles were rechromatographed (Table 8) to resolve vesicular from extra-vesicular $^{24}Na^{+}$. Aliquots of eluent were collected in 3 minute increments and analysed for $^{24}Na^{+}$ (Beckman 4000 gamma counter). The rate of $^{24}Na^{+}$ leakage was calculated as:

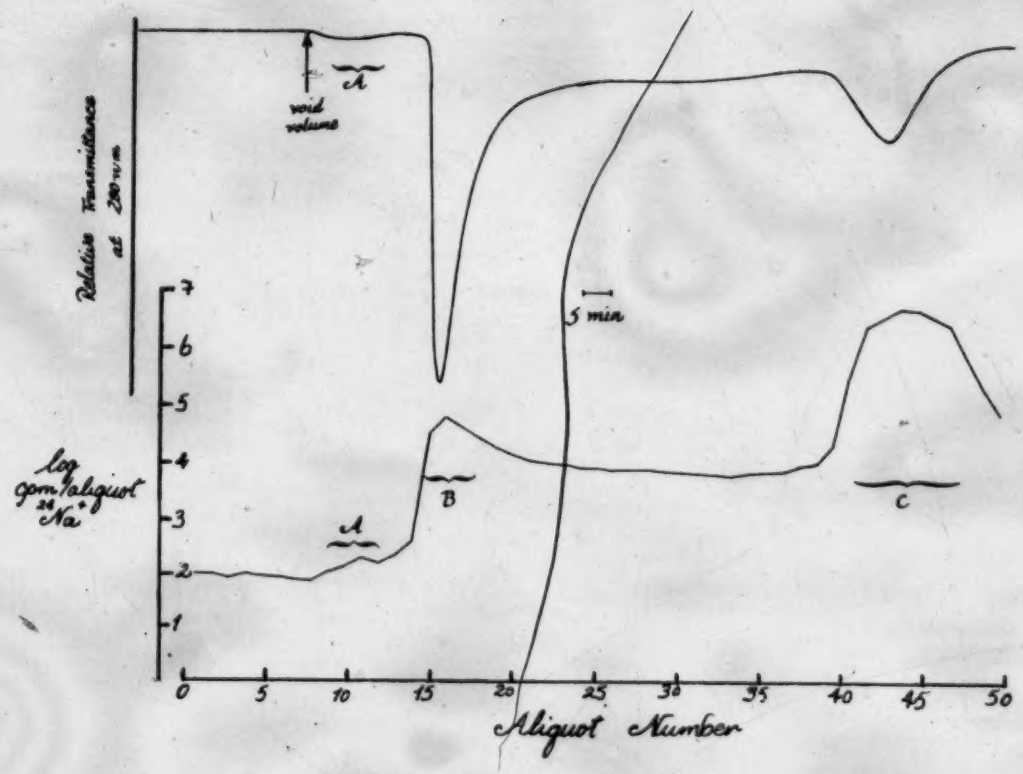


Figure VIII

Resolution of (A): multilamellar vesicles, (B): unilamellar vesicles and (C): extravesicular $^{24}Na^+$ in a sonicated RBC lipid suspension by absorbance UV spectroscopy at 280 nm and by gamma-spectroscopy of the eluent from a sepharose 4B column. The fractions containing the unilamellar vesicles were pooled and used for experiments.

$$\frac{\text{extravesicular } {}^{24}Na^{+} \text{ counts}}{\text{total } {}^{24}Na^{+} \text{ counts}} \times \frac{100\%}{t(hr)} = \frac{\%\ Na^{+} \text{ leakage}}{hr}$$

Leakage of $^{24}Na^{+}$ from RBC lipid vesicles was monitored during irradiation by flow dialysis. The 1-ml samples were dialysed against a 0.5 ml/min flow of the same buffer in which the vesicles had been sonicated (Table 4), excluding $^{24}Na^{+}$. Dialysis was not interrupted while the samples were maintained under control conditions, followed by irradiation, and finally returned to control conditions (Table 7). Aliquots of dialysate were collected every 3 or 5 minutes and then analysed for $^{24}Na^{+}$. The rate of leakage of $^{24}Na^{+}$ from the RBC lipid vesicles was determined by pooling the counts from the aliquots of dialysate collected during sham or microwave exposure. The portion of the total sample $^{24}Na^{+}$ leaked during each period was calculated and the resulting values were adjusted to $\%Na^{+}$ leakage/hour.

Table 7

The vesicle suspension exposure scheme for experiments V1 through V5.

sample	lipid	sample		irrad. sample		control sample	
chamber used	conc. (mg/ml)	size (ml)	T°C	exposure duration	SAR mW/g	duration	sequence
V1 2	2.5	8.5	25	6 h	1	6 h	same time
				9 h	1	9 h	same time
V2 2	2.5	8.5	25	6 h	1	6 h	same time
				9 h	1	9 h	same time
V3 3	2.5	2.0	23	11 h	25	11 h	same time
				19 h	25	19 h	same time
V4 1	1.12	1.0	23	36 min	2	33 min	before
						42 min	after
V5 1	1.12	1.0	23	70 min	2	60 min	before
						60 min	after

Table 8

Specifications of the post-exposure chromatographic procedures used to separate vesicles from leaked, extravesicular $^{24}Na^{+}$ after microwave exposure.

exp #	type	flow rate (ml/min)	void volume (ml)	column dimensions (cm)
V1	Sephadex G-25	3	31	50 x 1
V2	Sephadex G-25	3	31	50 x 1
V3	Sephadex G-25	3	15	25 x 1
V4	(leakage determined by flow dialysis)			
V5	(leakage determined by flow dialysis)			

2-3 : Calculation of Na^{+} Permeability

The trapped volume of the sonicated egg-PC vesicles was assumed to be the same as that described by Huang and Thompson (53): 0.9849 ml/g of egg-PC. It was predicted from the equation:

$$\frac{0.9849 \text{ ml trapped}}{\text{g egg PC}} \times \frac{0.025 \text{ g egg PC}}{\text{ml suspension}} \times 100$$

that 25% of the suspension would be trapped by the sonicated egg-PC vesicles. This figure agreed well with the 2.0%, 2.0% and 2.4% of the $^{24}Na^{+}$ trapped by the vesicles in experiments V1, V2, and V3 respectively. The outside diameter of the egg PC vesicles was taken to be the same as those of Huang and Thompson; 210±0.8 nm(52, 30).

Erythrocyte lipids were sonicated, as described, with 0.3 μCi ^{14}C-cholesterol and 12.5 μCi ^{3}H-mannitol. The vesicle suspension was chromatographed on a Sepharose 4B column (Table 6). The proportion of the original ^{14}C-cholesterol isolated in the vesicle peak times the original amount of lipid in the sonicated suspension was an index of the amount of lipid in the isolated vesicle population. Likewise, the proportion of the original ^{3}H-mannitol, a soluble but impermeant sugar, isolated in the vesicle peak times the volume of the original suspension provided the volume trapped by the above ^{14}C-vesicles. This determination yielded a value of 2.2 ml/g RBC-lipid for the trapped volume of sonicated RBC-lipid vesicles. This trapped volume corresponds to a vesicle population with a mean diameter of about 60 nm (27).

Using these values for the trapped volume and vesicle diameter the total bilayer area of the vesicles and moles $^{24}Na^{+}$ trapped per cm^2 of bilayer were calculated. The leakage rates in %/h could thus be converted to leakage rates in $mol/cm^2 \cdot h$ and hence the values of P_{Na} (cm/s) could be calculated.

3 : RESULTS AND DISSCUSSION

The variability among the $^{24}Na^{+}$ leakage rates from egg-PC vesicles (Table 9) reflects the relatively slow (~1%/h) rate of diffusion of $^{24}Na^{+}$ across the bilayer. Large counting errors were also associated with the low level of radioactivity in the aliquots of extravesicular $^{24}Na^{+}$ collected by chromatography

Table 9

The effect of microwaves on $^{24}Na^+$ leakage from sonicated vesicles of egg-PC and RBC lipid.

				control		irradiated	
experiment	frequency MHz	Temp ^oC	time (h)	Na^+ leakage (%/h $\pm S_{\bar{x}}$)	P_{Na^+} (cm/s)	Na^+ leakage (%/h $\pm S_{\bar{x}}$)	P_{Na^+} (cm/s)
V1(egg PC)	2350-2550	25	9	0.085 ± 0.004	2.99×10^{-14}	0.086 ± 0.051	3.03×10^{-14}
V2(egg PC)	2350-2550	25	9	0.158 ± 0.035	5.58×10^{-14}	0.150 ± 0.023	5.28×10^{-14}
V3(egg PC)	2450 CW	23	19	0.149 ± 0.039	5.25×10^{-14}	0.122 ± 0.021	4.31×10^{-14}
V4(RBC)	2450 CW	23	a	9.24 ± 0.81	2.02×10^{-11}	8.67 ± 0.91	1.90×10^{-11}
V5(RBC)	2450 CW	23	a	10.60 ± 0.93	2.32×10^{-11}	10.55 ± 0.94	2.32×10^{-11}

a - done by flow dialysis, for times see Table 7

of the samples (Figure IX) (30). It is assumed, however, that the variance is the same in all figures used to calculate the mean leakage rates as presented. The standard errors (SEM) of these means are therefore accurate (105) and the absence of a microwave effect on Na^+ leakage from egg PC vesicles at 25 ^oC, is statistically significant.

Counting error in Experiment V3 (Figure X) was minimised by increasing the counting time of each aliquot and performing four replicate counts of each aliquot (30). While these measures minimised machine error they had little effect on the standard errors of the two means compared with those in experiment V1 or V2. We must assume that the pooled means (0.131 ± 0.039 and 0.119 ± 0.032 $\%Na^+$ leaked/h from control and exposed vesicles, respectively) accurately reflect the mean and variability of Na^+ leakage from different populations of egg-PC vesicles (Table 9). As in the two previous experiments, microwaves did not affect Na^+ leakage although these vesicles were exposed to microwaves for periods of up to 19 h at 23 ^oC (Figure XI).

The $^{24}Na^+$ leakage rates from RBC lipid vesicles (Table 9) were less variable than $^{24}Na^+$ leakage rates from egg PC vesicles. This was due to the much higher $^{24}Na^+$ leakage from RBC lipid vesicles (Table 9). Furthermore, the procedure used to determine the leakage rates eliminated most of the machine error. Leakage of $^{24}Na^+$ from RBC-lipid vesicles was monitored by flow dialysis (Figure XII) and the rates were calculated by integrating cpm/aliquot over a minimum of twelve 3-min aliquots. This integration averaged out any deviation that resulted from

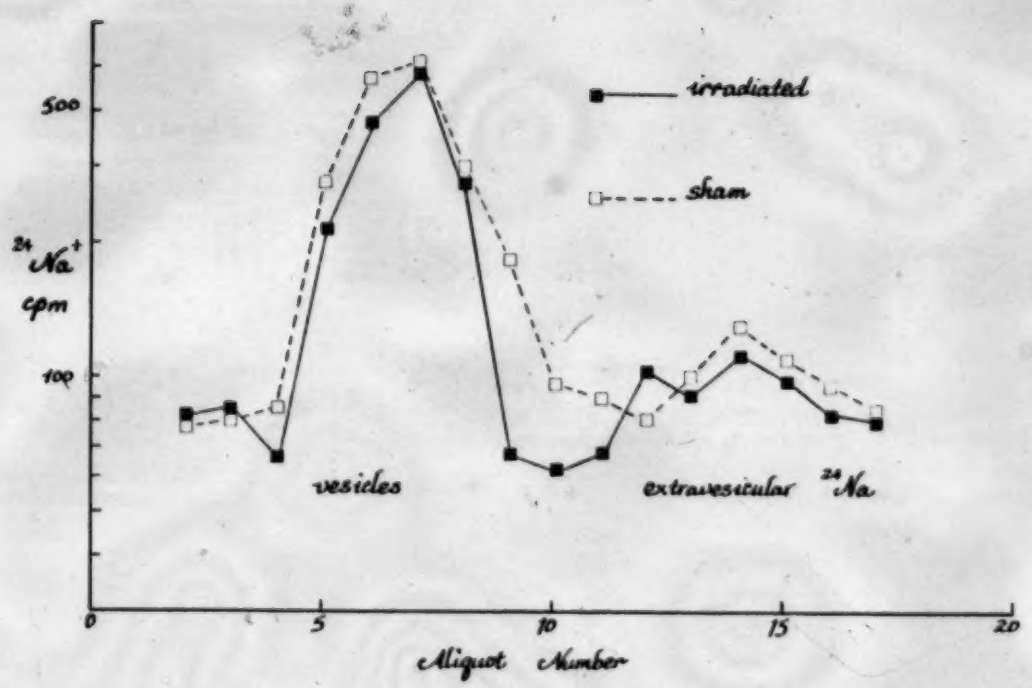


Figure IX

The effect of microwaves on $^{24}Na^+$ leakage from sonicated egg-PC vesicles in experiment V3 shown by Sephadex G25 chromatography of the vesicle sample after 19 h of irradiation.

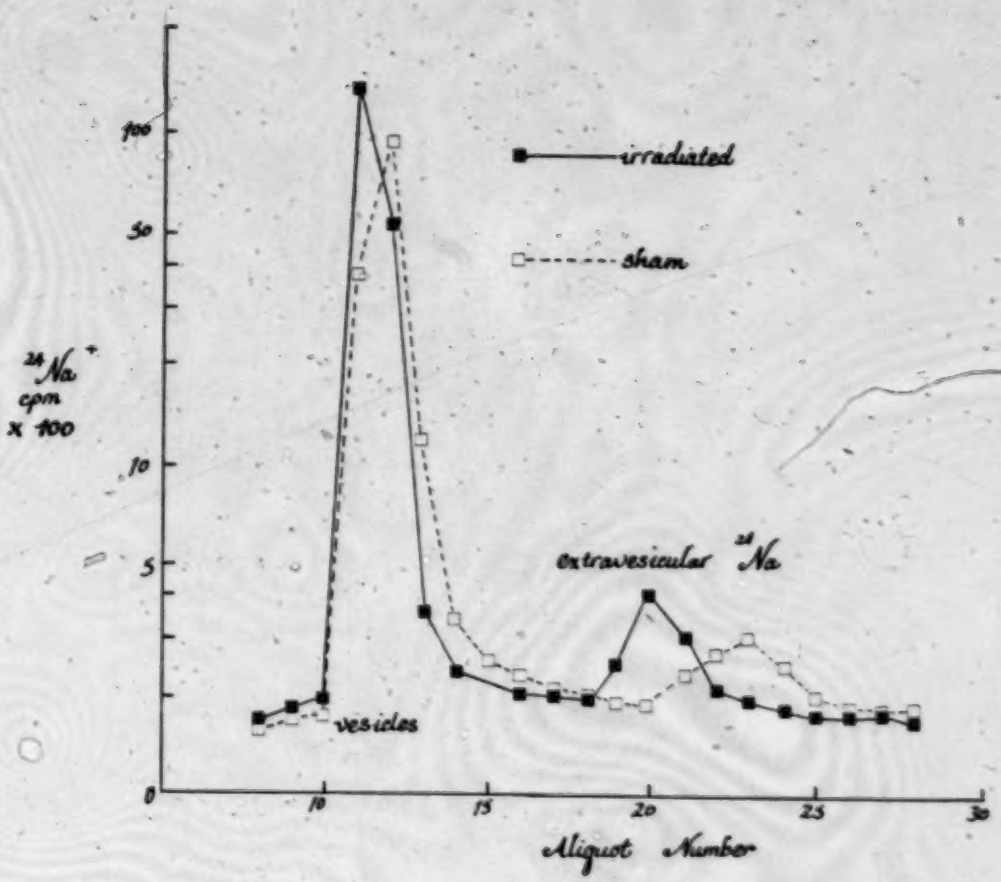


Figure X

The effect of microwaves on $^{24}Na^{+}$ leakage from sonicated egg PC vesicles in experiment V1 shown by Sephadex G25 chromatography of the vesicle sample after 9 h of irradiation.

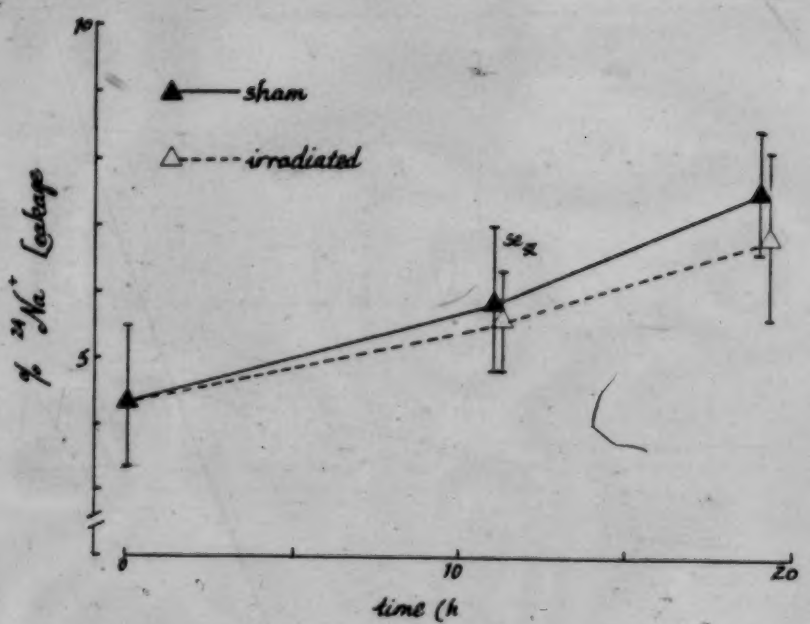


Figure XI

The effect of microwaves on $^{24}Na^{+}$ leakage from sonicated egg-PC vesicles in experiment V3.

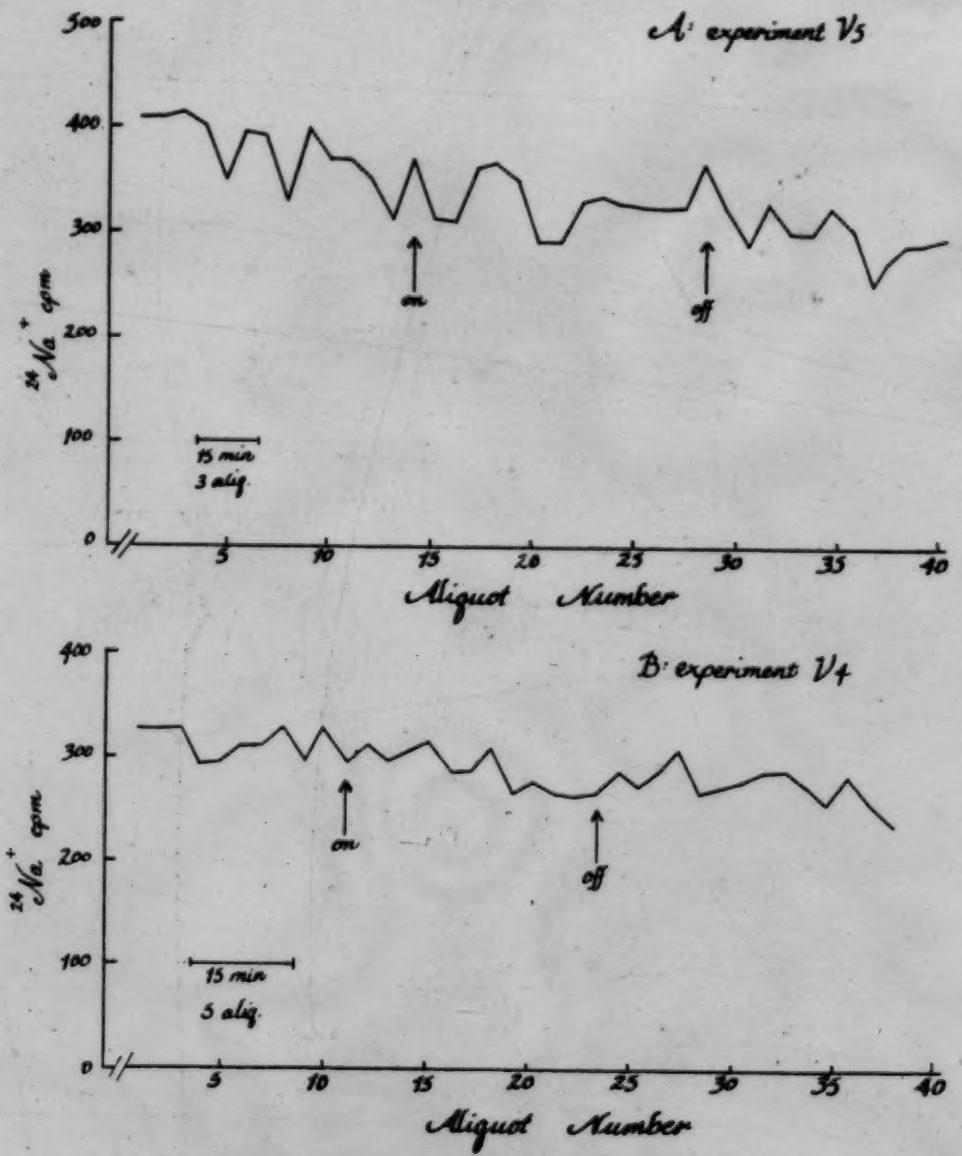


Figure XII

The effect of microwaves on the cumulative $^{24}Na^+$ leakage from RBC-lipid vesicles measured by flow dialysis of suspensions of sonicated RBC-lipid vesicles. (A): experiment V5, (B): experiment V4. The onset (on) and termination (off) of irradiation are shown for each experiment.

counting error. Low-level 2450-MHz-CW microwaves exerted no apparent effect on the rate of $^{24}Na^{+}$ leakage, calculated as described above (Table 9), or as monitored by flow-dialysis (Figure XII).

The reported range of Na^{+} leakage rates from egg-PC vesicles, 0.085-0.158%/h, lie within the range described by Papahadjopoulos (84) for sonicated DPPC (dipalmytoyl phosphatidylcholine) vesicles: 0-0.5%/h. Further, he showed that Na^{+} leakage from PC vesicles is relatively constant below 37 ^{o}C. No detectable microwave-induced temperature increases ($\pm$ 0.1 ^{o}C), that could have caused an increase in $^{24}Na^{+}$ leakage, were noted in any of the exposed samples maintained at temperatures between 23 and 25^{o}C. The range of leakage rates reported for DPPC vesicles may not be strictly comparable to egg-PC vesicles, which contain a number of different fatty acids (45), since acyl chain composition is known to affect the Na^{+} permeability of vesicles (85). The calculated Na^{+} permeabilities for the egg PC vesicles (Table 9) is within that previously reported for egg PC vesicles: 1.2×10^{-14}cm/s (57) to 2.8×10^{-12} cm/s (85). The $P_{Na^{+}}$ values are also of the same order as those reported for other PC containing vesicles: 5 to 11×10^{-14}cm/s (84).

Although phosphatidylcholine is a major constituent lipid of the erythrocyte membrane (56) and provides a good initial model of a biological lipid bilayer, the permeability of many membranes is influenced by the proportions of different phospholipids and steroids (83, 85). In this respect unilamellar vesicles of

extracted RBC-lipid provide a more realistic and convenient model of the non-proteinaceous portion of erythrocyte membrane.

The permeability of the RBC lipid vesicles is three orders of magnitude higher than that of the egg-PC vesicles (Table 9). The increased leakage observed in the RBC-lipid vesicles over that of the PC vesicles is comparable to values reported elsewhere, i.e; that Na^+ leakage rates from lecithin vesicles are increased about 20x by adding equimolar concentrations of other lipids including cholesterol (65). RBC-lipid extract contains large amounts of cholesterol and a variety of lipids (90).

There are two possible explanations for the P_{Na+} of the RBC vesicles (Table 9) being an order of magnitude less than that reported for intact RBC membranes: 10^{-10} cm/s (57). The intact erythrocyte membrane is about 50% protein (75) and about half the lipid component is cholesterol (90). If one removes the protein, the lipid previously associated with the membrane protein (47, 114) is now free to interact with the cholesterol. Thus there may be an effective increase in the lipid:cholesterol ratio in the bilayer. The $P_{Na}+$ of mixed lipid vesicles has been shown to decrease with increasing cholesterol content (83).

The other possiblity is that the lower cation permeability of the RBC vesicles compared with that of intact erythrocytes results from the absence of protein in the RBC-lipid vesicles. Incorporating Glycophorin, a major protein of the erythrocyte membrane into vesicles of DOPC (dioleoyl phosphatidylcholine) has been shown to increase the leakage of K^+ by 76% (120). The possibility that the ouabain-poisoned (Na^++K^+)ATPase may

still mediate some form of cation transport has been suggested (8) and may also be responsible for the apparently high P_{Na^+} of the intact erythrocyte membrane.

Leakage of $^{24}Na^+$ from lipid vesicles is assumed to be by diffusion unless degradation of the vesicles is occuring. There are accounts of up to 52% of total Na^+ leakage from PC vesicles being due to vesicle degradation (65), but Paphadjopoulos (83) reports insignificant degradation of egg-PC vesicles prepared at 4 ^{o}C, by sonication under nitrogen. Further, the P_{Na^+} values for PC vesicles (Table 9) are at the low end of the range of reported P_{Na+} values for PC vesicles.

IV UNIDIRECTIONAL FLUXES

1 : INTRODUCTION AND PURPOSE

The lipid bilayer portion of the erythrocyte membrane provides not only the major permeability barrier between the intracellular and extracellular environments but also the supporting structural matrix for membrane proteins such as the Na^+ and K^+ translocating ATPase. Having established that low-level 2450-MHz microwaves do not significantly influence Na^+ leakage from sonicated RBC-lipid vesicles, the influence of microwaves on the $^{24}Na^+$ efflux (31) and $^{42}K^+$ influx mediated by the $(Na^+ + K^+)$ATPase in intact human erythrocytes was studied. Fluxes of Na^+ and K^+ mediated by the $(Na^+ + K^+)$ATPase were resolved as the difference in flux rates with and without ouabain. Fluxes occuring in the presence of ouabain probably differ from the Na^+ leakage from RBC-lipid vesicles because of the absence of protein in the latter. Ouabain-poisoned cells may effect cation flux by facilitated diffusion or by some active but ouabain-insensitive cation pump (50). The latter, ouabain-insensitive contributions must be considered in evaluating the influence of microwaves on Na^+ and K^+ transport across the intact red-cell membrane.

The temperature dependence of both active and passive Na^+ and K^+ transport processes may have special implications on the biological effects of microwaves. Olcerst et al (81) demonstrated

increased $^{22}Na^+$ efflux from ouabain-treated rabbit erythrocytes exposed to 2450-MHz microwaves only in the range of 8 to 13 °C and also at 22.5 °C and 38 °C. The authors correlated these temperatures with discontinuities in the Arrhenius plots for passive $^{22}Na^+$ efflux from rabbit erythrocytes. Therefore, temperature was introduced as an experimental variable in the following experiments. Temperature dependence of the effect of microwaves on Na^+ and K^+ transport and other biological systems (29) may prove to be the cause of conflicting reports (7, 31, 44, 53, 54, 67, 81) concerning the existence of a biological effect in this system.

2 : MATERIALS AND METHODS

2-1 : Na^+ Efflux - Cell Suspension

Venous blood (~100 ml) was drawn from an apparently healthy human volunteer 24 hours before the start of each experiment. The blood was collected in two tubes, each containing 10-ml acid:citrate:dextrose (75 mM Sodium Citrate, 38 mM citric acid and 136 mM dextrose) and was immediately centrifuged at 2500 x g for 5 min. The erythrocytes were separated, suspended in 3 vol of chilled 'high K^+ buffer' (2.5 mM Na_2HPO_4, 8.55 mM NaCl, 140 mM KCl and 1 mM $MgCl_2$ adjusted to pH 7.4), and recentrifuged at 2500 x g for 5 min. This washing procedure was repeated twice with the 'high K^+ buffer' to remove excess Na^+ before loading the cells with $^{24}Na^+$.

The cells were incubated at 4 °C for 20 h in 2 vol of 'loading buffer' ['high K^+ buffer' with the addition of 0.1 mM

paracholormercuribenzene sulfonate (PCMBS; Sigma Chemical Co., St. Louis, MI)]. The PCBMS was added to inhibit active Na^+ transport and increase Na^+ permeability of the cells (36). In this 'loading buffer', the 8.55 mM NaCl was replaced with 8.55 mM $^{24}NaOH$ (~31.1 μCi/mg; University of Alberta Slowpoke Facility) and the pH was adjusted to 7.4 with HCl. At the end of the incubation intra- and extracellular $^{24}Na^+$ concentrations had equilibrated. The $^{24}Na^+$-loaded erythrocytes were separated by centrifugation, 2500 x g for 5 min, and resuspended in 3 vol of 'clearing buffer' (2.5 mM Na_2HPO_4, 145 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 11 mM D-glucose and 2 mM L-cysteine (Sigma) at pH 7.4. L-Cysteine competitively binds PCMBS thereby disinhibiting the $(Na^+ + K^+)$ATPase and restoring the original P_{Na^+} of the cells (36, 116). The suspension was incubated at 37 °C for 1 h, during which time the clearing buffer was replaced twice. This procedure removed all of the PCMBS (37) and extracellular $^{24}Na^+$.

The $^{24}Na^+$-loaded cells were resuspended in 2 vol 'experimental buffer' ('clearing buffer' but without L-cysteine) and separated by centrifugation (2500 x g for 5 min). This washing procedure was repeated twice. The cells were resuspended to a hematocrit in the range of 30 to 50%. Half of the preparation was divided into 1-ml samples for determination of total Na^+ efflux. Ouabain (10^{-4} M, Sigma) was added to the other half; this was divided into 1-ml samples for the determination of ouabain-insensitive $^{24}Na^+$ efflux. All samples were stored at 4 °C until used.

2-2 : Na^+ Efflux-Experimental Procedure

Sample chamber #3 (Figure VI) was used for all Na^+ efflux experiments. A piece of dialysis membrane (spectrapore, 2 to 3000 MW) divided the sample and dialysate spaces for flow dialysis. Dialysis was started immediately after placement of the sample into the chamber. To insure that the cells were evenly distributed throughout the suspension during irradiation, water-saturated air was bubbled through the sample via the lower sample access port at 1.5 ml/min. Continuous mixing also insured the absence of thermal gradients within the sample caused by heat flow from the water jacket or potentially uneven microwave heating. The mechanical stress on the cells at this flow rate caused less than 1% hemolysis. The temperatures of the exposed samples measured before irradiation were the same as those measured after irradiation. These are reported, for each irradiation, in the results. The temperatures of the control samples, monitored during irradiation, did not deviate more than 0.05 ^{o}C during a run.

The dialysate (experimental buffer) flowed continuously via the dialysate ports and across the dialysis membrane at 30 ml/h and was collected every 5 min (12 x 2.5 ml/h) on a fraction-collector (LKB/Bromma 2112 Redirac). The $^{24}Na^+$ activity of each fraction was analysed on a Beckman 4000-gamma counter. Experimental measurements were taken only after the system had been allowed to equilibrate for 30 min.

In all other experiments, the sample was isolated from the

dialysate space of the sample chamber by a piece of Parafilm inserted in place of the dialysis membrane. The sample was inserted and allowed to equilibrate to the selected temperature for 30 min, then a 0.3-ml aliquot was withdrawn for determination of initial intra- and extracellular $^{24}Na^+$. After further incubation for 1 or 2 h, the remaining 0.7 ml was used for three replicate determinations (0.2-ml each) of the final intra- and extracellular $^{24}Na^+$ levels and for measurement of hematocrit.

To determine intra- and extracellular $^{24}Na^+$, 0.2 ml of 'experimental buffer' was layered on top of 0.2 ml dibutyl phthalate (43) in a 1.5-ml disposable Eppendorf centrifuge tube and an aliquot of the suspension was added. The replicate tubes were immediately centrifuged at 12,000 x g for 1 min. The upper aqueous layer was pipetted into another tube, and $^{24}Na^+$ activity of each component was determined on the gamma-counter. The Na^+ flux rate was calculated as meq Na^+/litre of packed cells per h (meq Na^+/ℓ cells·h) from the timed changes in the proportion of total $^{24}Na^+$ in the extracellular fluid or dialysate. Calculations were based on an intracellular Na^+ concentration of 20 meq Na^+/ℓ cells.

Two sets of experiments were performed with the isolated (non-dialysed) erythrocyte samples. In experiments 1 through 5 a different temperature, in the range of 18 to 35 °C, was selected for each of the five suspensions, and several samples of each suspension were incubated at that temperature. In experiments 6 to 8, samples of the same suspension were incubated at various temperatures. In all experiments, control and irradiated samples

were run simultaneously.

2-3 : Na^+ Efflux - Neutron Activation Analysis for Hg^{++}

The reversible inactivation of the $(Na^+ + K^+)$ATPase and increased Na^+ permeability of the erythrocyte membrane treated with PCMBS relies on the binding of Hg^{++} to protein sulfhydryl groups in the membrane. Although the effects of PCMBS on these membrane functions have been found to be completely reversible (36, 104) it was deemed necessary to establish, experimentally, the absence of Hg^{++} after washing with cysteine. Any remaining Hg^{++} could have complicated the accurate assessment of microwave bioeffects on Na^+ and K^+ transport.

Cells treated with 0.1 mM PCMBS, then washed with 2 mM cysteine, were assayed for Hg^{++}. 'White' erythrocyte ghosts were prepared (22, 24) and concentrated by centrifugation (15 min at 75,000 x g). Any remaining Hg^{++} was likewise concentrated, enhancing the sensitivity of the determination. This procedure also minimized the water content of samples to be exposed to high neutron fluxes, reducing the degree of radiolysis and hence the risk of high temperatures and pressures in the sample container during neutron activation.

The ghosts were activated in 200μl aliquots at the University of Alberta Slowpoke Facility by exposing them for 2 h to a neutron flux of $10^{12}/cm^2 \cdot s$. After cooling for 2 days, each of the ghost samples and simultaneously activated standards was analysed for 30 min on a Nuclear Data ND 660 multichannel analyser with an ORTEC WIN 15 coaxial Ge(Li) detector. The

amount of Hg^{++} was calculated from the 78 KeV gamma-decay peak of ^{197}Hg.

2-4: K^+ Influx - Experimental Procedure

The procedure used to study the influence of microwaves on $^{42}K^+$ influx is much simpler than that used to study $^{24}Na^+$ efflux. Influx measurement precludes complicated loading techniques.

Venous blood (~30 ml) was drawn from an apparently healthy human volunteer immediately before the start of the experiment and was centrifuged at 2500 x g for 5 min. The erythrocytes were resuspended in 3 vol of chilled buffer containing 2.5 mM Na_2HPO_4, 145 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$ and 11 mM dextrose at pH = 7.4 and centrifuged at 2500 x g for 5 min. This washing procedure was repeated twice. The cells were resuspended to a hematocrit of 23% in the same buffer (pH adjusted to 7.4 with HCl) but in which the K^+ was as $^{42}K^+$ (3.2 μCi/mg ^{42}KOH, supplied by the University of Alberta Slowpoke Facility). Ouabain was added at 10^{-4} M to one half of this suspension. Both suspensions were divided into 1-ml aliquots in 1.5 ml Eppendorf centrifuge tubes and stored at 4 ^{o}C until required.

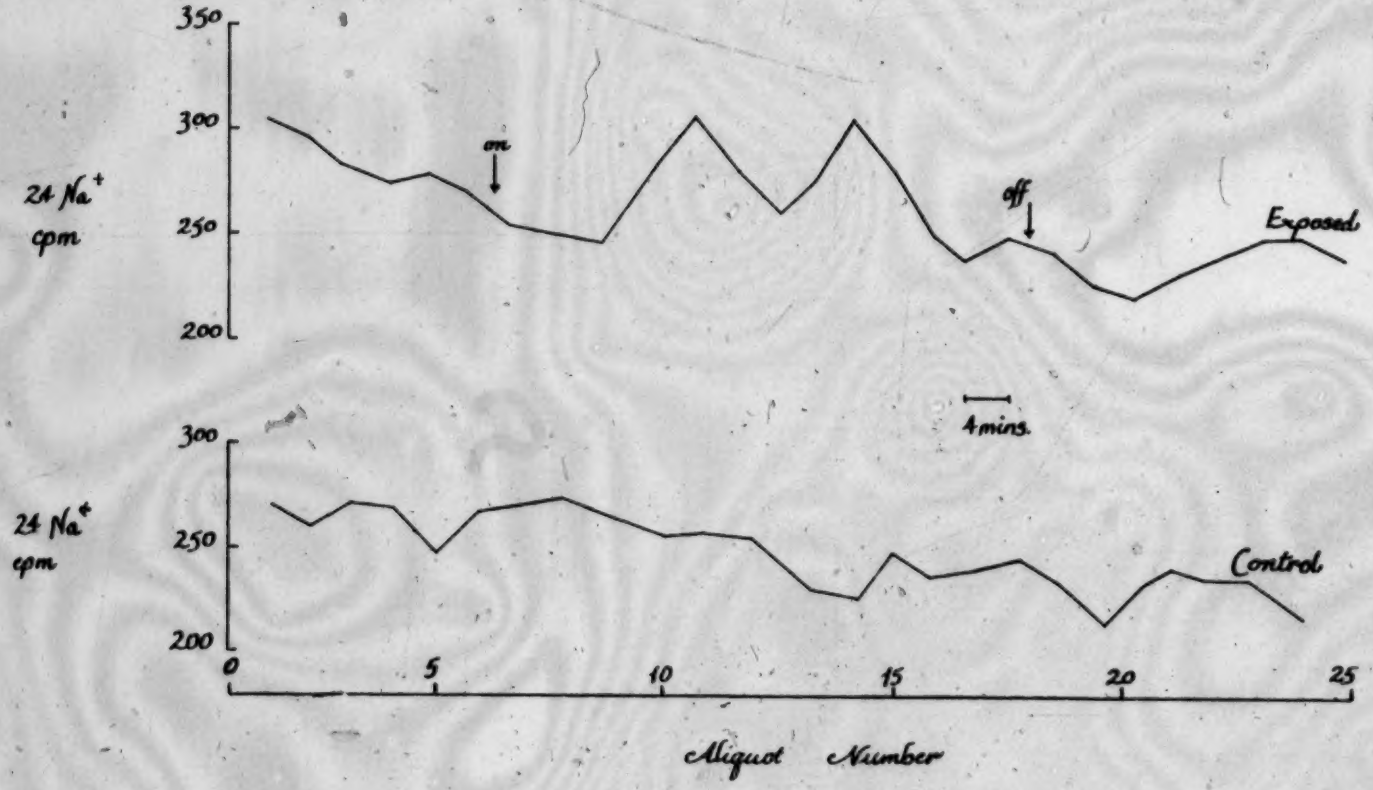
Samples were irradiated in pairs, one with and the other without ouabain, for 2 hr at the prescribed waterbath temperature. An equilibration period was not found necessary in these experiments. The samples were pre-warmed to the desired temperature by submerging the tubes in the waterbath before being

placed into the sample holder. This was one of the reasons for developing and using sample chamber #4 (Figure VII) for the K^+ influx experiments. Prewarming the samples used for Na^+ efflux experiments was never as effective since the sample chamber (#3, Figure VI) was fixed to the waveguide wall. Prewarmed samples always cooled in the access tubes leading to the chamber.

After the 2 h incubation period each of the irradiated and control samples was divided into 3 x 0.3-ml replicate samples for intra- and extracellular $^{42}K^+$ determination. The cells and buffer were separated and analysed for $^{42}K^+$ by the same procedure described previously for Na^+. Potassium influx rates were calculated from the proportion of $^{42}K^+$ inside the cells after 2 h incubation and were based on an extracellular K^+ concentration of 4 mM.

3 : RESULTS AND DISCUSSION

Direct determination of cellular and extracellular $^{24}Na^+$ and $^{42}K^+$ was more effective than flow dialysis in accurately determining the effect of microwaves on Na^+ and K^+ fluxes in human erythrocytes. No significant effect of microwave exposure on Na^+ efflux from human erythrocytes was resolved by flow dialysis by the method and system described above (Figure XIII). At the hematocrits, temperatures and sample volumes used, the absolute Na^+ efflux rates--typically 1.2×10^{-3} meq Na^+/h at 23 ^{o}C--were such that a slow dialysate flow rate was required to accumulate a moderately reproducible number of counts per aliquot (at 2 x background activity the standard deviation is



hc = 36%

Figure XIII

The effects of microwaves on $^{24}Na^{+}$ efflux from human erythrocytes as revealed by flow dialysis of an exposed suspension of cells (hct =36%, 37500 cpm/sample). The onset (on) and termination (off) of irradiation are indicated.

~15%). The low $^{24}Na^+$ activities of each aliquote collected were further complicated by errors introduced due to the pulsing behavior of the low-speed peristaltic pump driving the dialysate. Within the constraints of these errors, however, the rate of Na^+ efflux was shown to be relatively constant (2.45 $\pm$ 0.28 meq Na^+/l cells·h at 23 °C) at 5 min intervals over the 2-h of incubation. Direct measurement of cellular $^{42}K^+$ revealed a linear rate of accumulation and thus a constant rate of $^{42}K^+$ influx to erythrocytes over 3 h (Figure XIV). Thus, the magnitude and kinetics of Na^+ and K^+ transport in human erythrocyte suspensions are such that their flux rates may be accurately estimated by measuring isotope concentration differences in 1 to 2 h increments. Faster kinetics or higher enzyme concentrations would have precluded use of this technique since the accumulation of $^{42}K^+$ and reduction of $^{24}Na^+$ would have affected the apparent flux rates within the incubation period.

Albeit insignificant, the flow dialysis data show a trend thatindicates increased Na^+ efflux from cells exposed to low-level 2450 MHz microwaves (Figure XIII). To confirm this trend the next five experiments (1-5, Table 10) were not based on flow dialysis; $^{24}Na^+$ leakage was determined by the direct method described above. In experiments 1 through 5 each blood sample was from a different donor and each sample was incubated at a different temperature. Microwaves influenced total Na^+ efflux from cells incubated at 20.1, 23.0 and 24.0 °C but not from those incubated at 18.6 or 34.7 °C (Table 10). The increase in

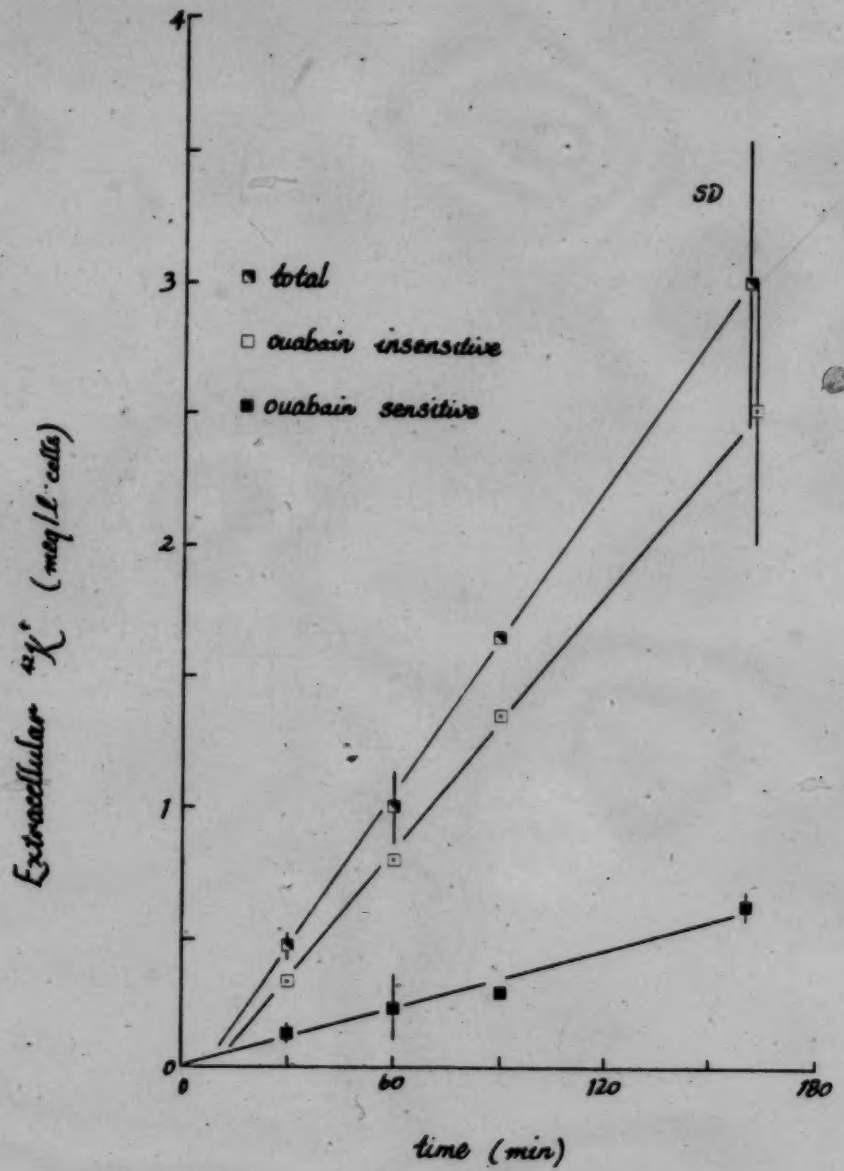


Figure XIV

Time course of the accumulation of $^{24}K^+$ by human erythrocytes incubated at 25 °C with and without 0.1 mM ouabain. Accumulation by ouabain sensitive processes derived by subtraction of that accumulated in the presence of ouabain from that in the absence of ouabain. The slopes of these lines indicate K^+ influx rates of: 1.25 meq/ cells·h total, 0.22 meq/ cells·h ouabain-insensitive and 1.03 meq/ cells·h ouabain-sensitive.

Table 10

The effects of low-level 2450-MHz microwaves on total, ouabain-insensitive and ouabain-sensitive Na^+ efflux from human erythrocytes. A: results of the first set of experiments done on blood samples from different donors each done at different single temperatures. B: results of the second set of experiments done on blood samples from different donors, each done at a number of temperatures.

				meq Na^+ efflux / litre cells · h ± SE (n)					
				total		ouabain insensitive		ouabain sensitive	
expt. #	temp. °C	hct. %	SAR mW/g	sham	irradiated	sham	irradiated	sham	irrad.
1	18.6	40	3.3	1.282±0.031(4)	1.260±0.023(4)	1.141±0.021(4)	1.094±0.020(4)	0.141	0.166
2	20.1	43	3.3	2.144±0.015(16)	2.349±0.016(4)	1.415±0.043(12)	1.773±0.032(4)	0.729	0.579
3	23.0	40	2.9	2.234±0.023(4)	2.782±0.022(4)	1.535±0.022(4)	2.379±0.052(4)	0.700	0.043
A 3b	23.0	40	2.9	2.290±0.021(4)	2.275±0.024(4)				
4	24.0	34	3.0	2.536±0.049(4)	2.885±0.0[illegible](4)	1.700±0.046(4)	2.336±0.023(4)	0.836	0.519
4b	24.0	34	3.0	2.503±0.040(4)	2.614±0.025(4)				
5	34.7	38	3.2	4.180±0.050(2)	4.400±0.194(4)	1.455±0.055(4)	1.353±0.039(3)	2.726	3.048
6	17.3	40	2.6	0.987±0.043 a	0.868±0.070(3)				
6	21.3	37	2.5	1.644±0.043 a	2.530±0.187(3)				
6	25.0	42	2.5	2.617±0.043 a	2.534±0.247(3)				
7	19.6	47	2.3	1.039±0.114(2)	1.222±0.034(3)	0.755±0.041 a	0.860±0.129(3)	0.284	0.262
B 7	21.8	45	2.4	1.446±0.034 a	1.802±0.016(2)	0.979±0.041 a	1.387±0.133(3)	0.467	0.415
7	24.0	42	2.3	2.046±0.077(3)	2.248±0.043(3)	1.313±0.041 a	1.325±0.042(3)	0.733	0.923
8	21.4	39	1.9	1.381±0.021 a	1.733±0.048(3)	1.039±0.039 a	1.471±0.032(3)	0.342	0.262
8	25.5	48	1.7	2.446±0.031(3)	2.658±0.058(3)	1.414±0.039 a	1.457±0.072(3)	1.032	1.201
8	30.8	43	1.6	2.999±0.054 a	3.154±0.029(3)	1.500±0.039 a	1.592±0.034(3)	1.499	1.562

a - estimates obtained from regression analysis of control values
b - neither sample irradiated

the total Na^+ efflux from irradiated cells compared with controls within the effective temperature range appeared to be the result of an increase in the ouabain-insensitive Na^+ and decrease in the ouabain-sensitive Na^+ efflux (Figure XV). The maximal change observed in this set of experiments was at 23 °C. The ouabain-insensitive flux increased ~50% and the ouabain-sensitive flux decreased ~40% resulting in a 25% increase in the total Na^+ efflux (Figure XV).

Cell suspensions incubated at 23.0 °C and 24.0 °C within the waveguide system but not irradiated, showed total Na^+ efflux rates of 2.275 ± 0.024 and 2.614 ± 0.025 meq/ℓcell·h respectively (Table 10). These values were not significantly different from the total Na^+ efflux rates of cells incubated at 23.0 °C and 24.0 °C in the control-sample chambers: 2.290 ± 0.021 and 2.503 ± 0.040 meq/ℓ cell·h, respectively. The observed differences in Na^+ efflux are therefore a result of microwave irradiation and not other differences in the incubating environment.

The differences in magnitude and direction of the microwave effects on ouabain-sensitive and -insensitive Na^+ fluxes from erythrocytes suggest interactions of the microwaves with more than one of the Na^+ flux pathways. Applying a 2-way analysis of variance (ANOVA) (105) to the Na^+ efflux data from cells incubated in the effective temperature range reveal this difference as shown in Table 11. Statistical analysis showed the significant and well documented effects of ouabain (25, 38, 59, 74) and also showed the effects of microwaves on Na^+ efflux

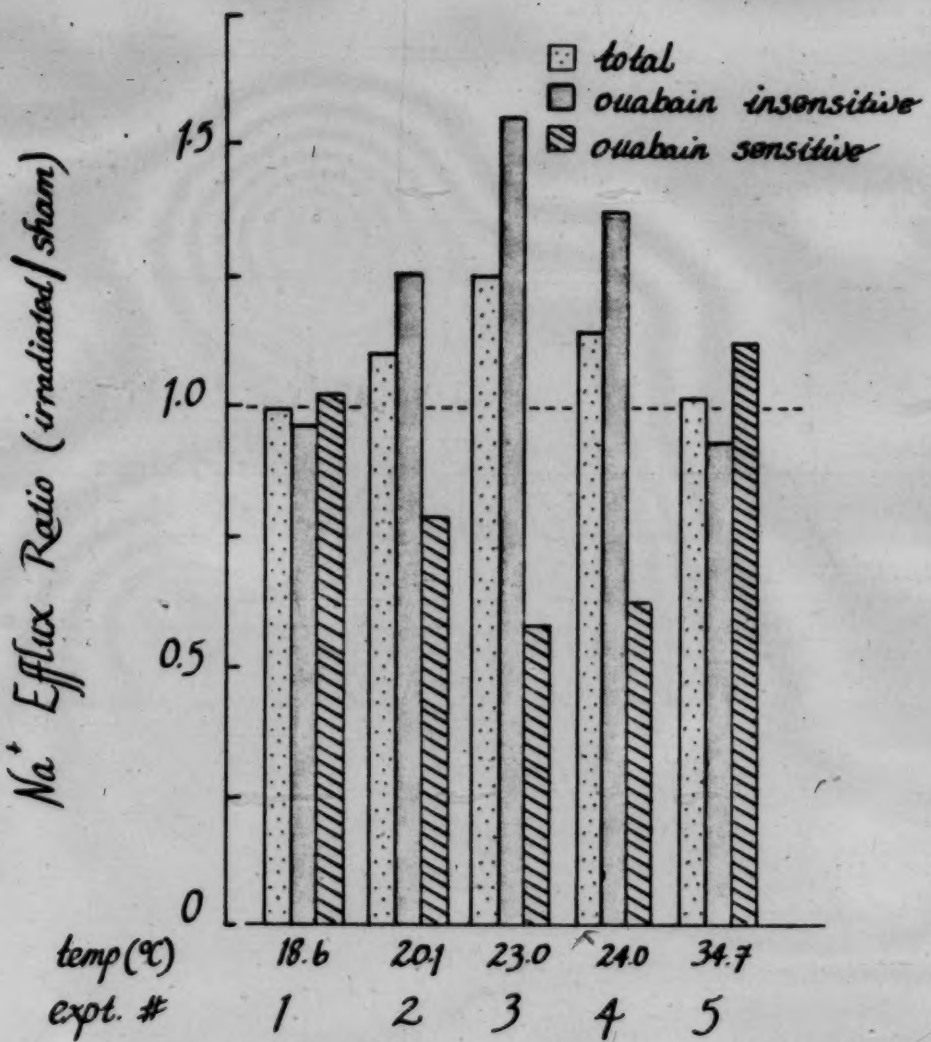


Figure XV

The influence of microwaves on Na^+ efflux from human erythrocytes. For each of these experiments cells from different donors were used and were incubated at single, discreet temperatures.

with and without ouabain (Figure XV). The significant degree of interaction (.001 < P < .01) between the ouabain and microwave effects on Na^+ efflux preclude simple additivity as the cause of the difference in the microwave effect in the presence and absence of ouabain.

Table 11

Statistical analysis of the influence of microwaves of ouabain, and of both on the Na^+ efflux from human erythrocytes.

Temp. °C	ouabain	Na efflux meq/ℓ cells.h control±SE(n)	Na efflux meq/ℓ cells.h irrad.±SE(n)	2 x 2 ANOVA subgroup	2 x 2 ANOVA P<
20	absent	2.144±0.015(16)	2.349±0.016(4)	A	.001
				B	.001
	0.1 mM	1.415±0.043(12)	1.773±0.032(4)	AxB	.001
23	absent	2.235±0.023(4)	2.782±0.022(4)	A	.001
				B	.001
	0.1 mM	1.535±0.022(4)	2.379±0.052(4)	AxB	.001
24	absent	2.536±0.049(4)	2.885±0.061(4)	A	.001
				B	.001
	0.1 mM	1.700±0.046(4)	2.366±0.023(4)	AxB	.01

A - significance of interaction of microwaves with efflux
B - significance of interaction of ouabain with efflux
AxB - significance of interaction of the effects of microwaves and ouabain combined. The null hypothesis being that the effects are additive.

Three more experiments were designed to more accurately resolve the temperature specificity and magnitude of the

microwave effect on Na^+ efflux. In each of this second set of experiments, samples of the same cell suspension were incubated at different temperatures. This approach eliminated individual differences between samples and allowed a more accurate delineation of the effective temperature range. These experiments, typified by the results presented in Figure XVI, confirm observations made in the first set of experiments (Table 10).

Analysis of the combined results of all Na^+ efflux experiments, divided into three temperatures ranges, 10 to 19.9 ^oC, 20 to 24.9 ^{o} and 25 to 40 ^oC (Figure XVII) revealed significant microwave effects only in the 20 to 24.9 ^oC range. Microwaves were associated with a mean increase of 23.1 ($\pm$ 5.8)% in total, a mean increase of 33.2 ($\pm$ 7.6)% in ouabain-insensitive and a mean decrease of 18.2 ($\pm$ 10.0)% in ouabain-sensitive, Na^+ efflux from all the human erythrocyte suspensions studied.

The influence of microwaves on total K^+ influx (Table 12) was not consistent with the microwave effects on Na^+ efflux (Table 10). Although the temperature specificity of the microwave effect on K^+ influx (Figure XVIII) was the same as that for the effect on Na^+ efflux (Figure XVII), microwaves caused a <u>decrease</u> in the total K^+ influx in the effective temperature range. The microwave-induced decrease in ouabain-sensitive K^+ influx (-0.074 $\pm$ 0.027 meq/ℓcell·h) exceeds the observed increase in ouabain-insensitive K^+ influx (+0.032 $\pm$ 0.024 meq/ℓcell·h) (Figure XIX) and consequently accounts for the

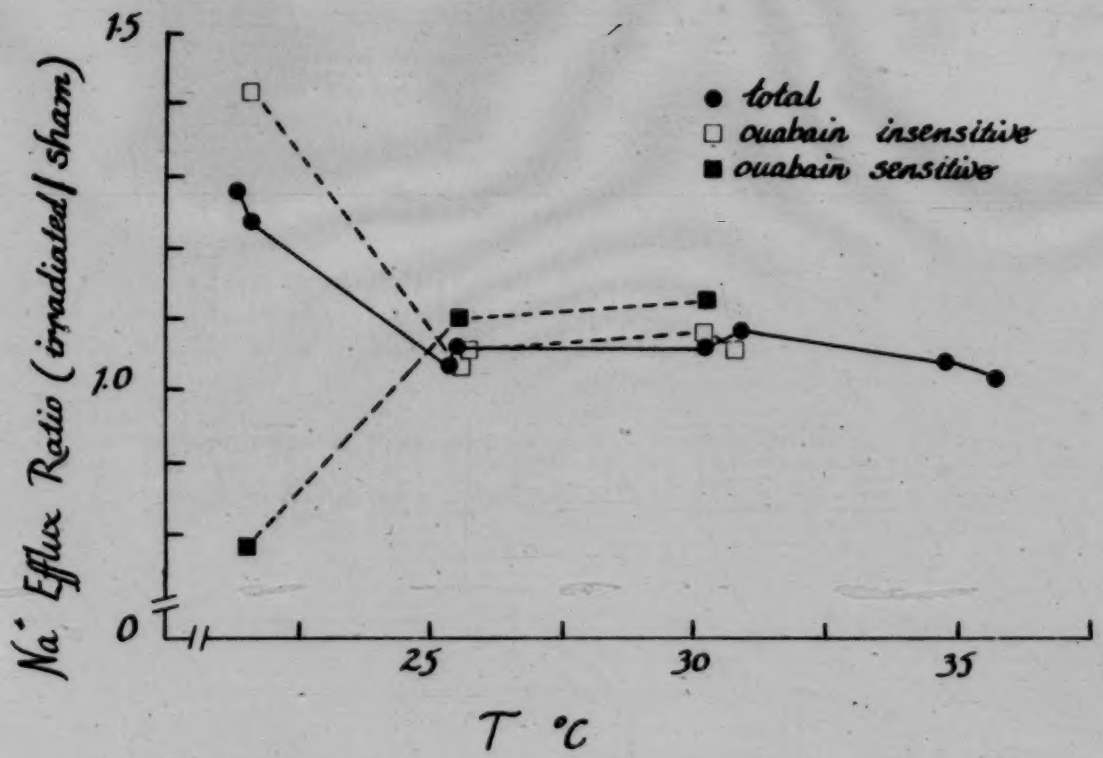


Figure XVI

The effect of microwaves and incubation temperature on Na^+ efflux from the erythrocytes of a single donor.

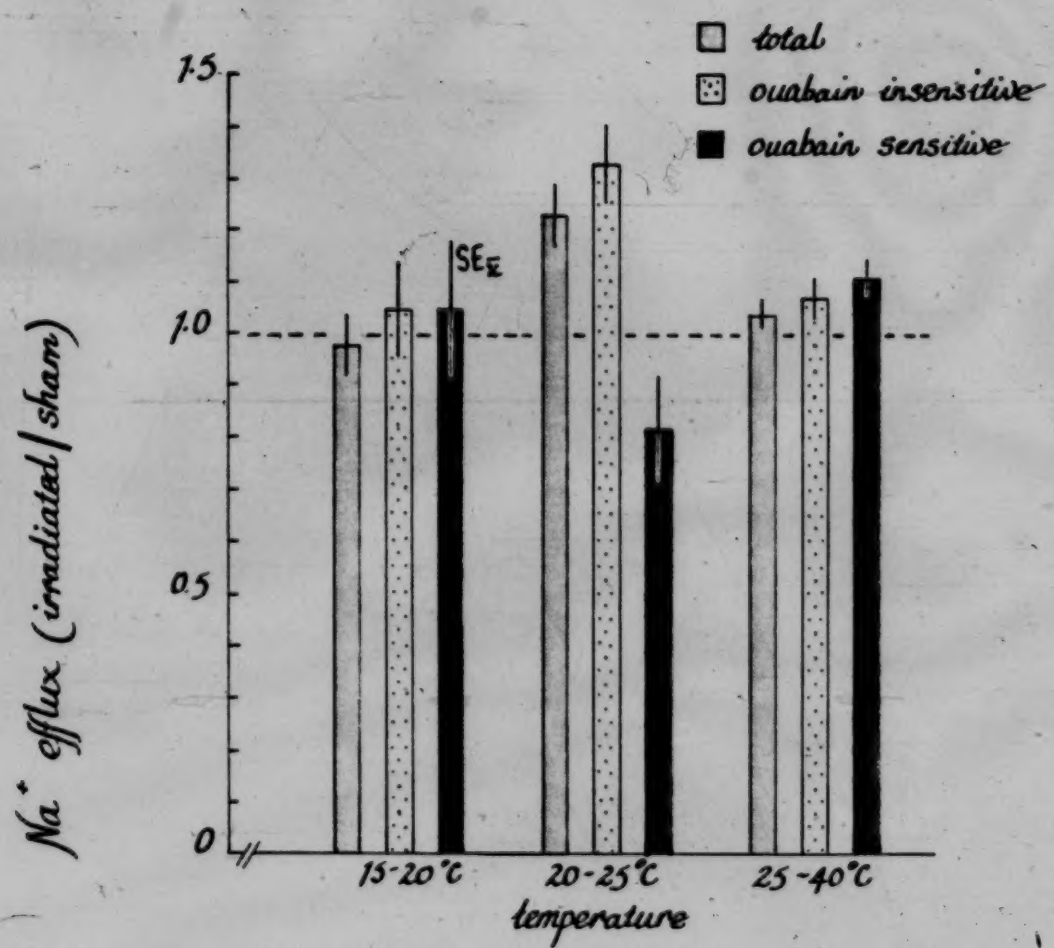


Figure XVII

The influence of microwaves on Na^+ efflux from human erythrocytes in different incubation temperatures ranges. Data from all Na^+ experiments were pooled for this comparison.

Table 12

The effect of low level (SAR = 3.0 mW/ml) 2450 MHz microwaves on total, ouabain-sensitive and ouabain insensitive K^+ influx in human erythrocytes (hematocrit = 23%). Each value is the mean of 3 replicate determinations ± SE

	meq K^+ influx / litre cells · hr					
	total		ouabain - insensitive		ouabain - sensitive	
Temp. C	sham	irrad	sham	irrad	sham	irrad
12.0	0.030±0.004		0.000		0.030±0.004	
13.5	0.125a	0.139±0.016	0.005a	0.017±0.004	0.107a	0.121±0.011
17.3	0.341±0.019		0.032±0.003		0.308±0.017	
18.7	0.447a	0.433±0.004	0.062a	0.061±0.011	0.367a	0.373±0.009
21.0	0.586±0.023		0.106±0.008		0.480±0.016	
21.8	0.627a	0.662±0.022	0.126a	0.139±0.011	0.482a	0.464±0.011
23.6	0.907±0.016	0.828±0.030	0.167±0.016	0.219±0.048	0.740±0.000	0.611±0.019
29.3	1.828±0.042		0.277±0.013		1.550±0.054	
29.9	1.882a	1.915±0.034	0.293a	0.364±0.013	1.586a	1.551±0.023
34.1	2.304±0.019		0.392±0.018		1.867±0.042	
36.1	2.469a	2.544±0.048	0.441a	0.407±0.016	1.996a	2.120±0.048

a - estimates obtained from regression analysis of control values

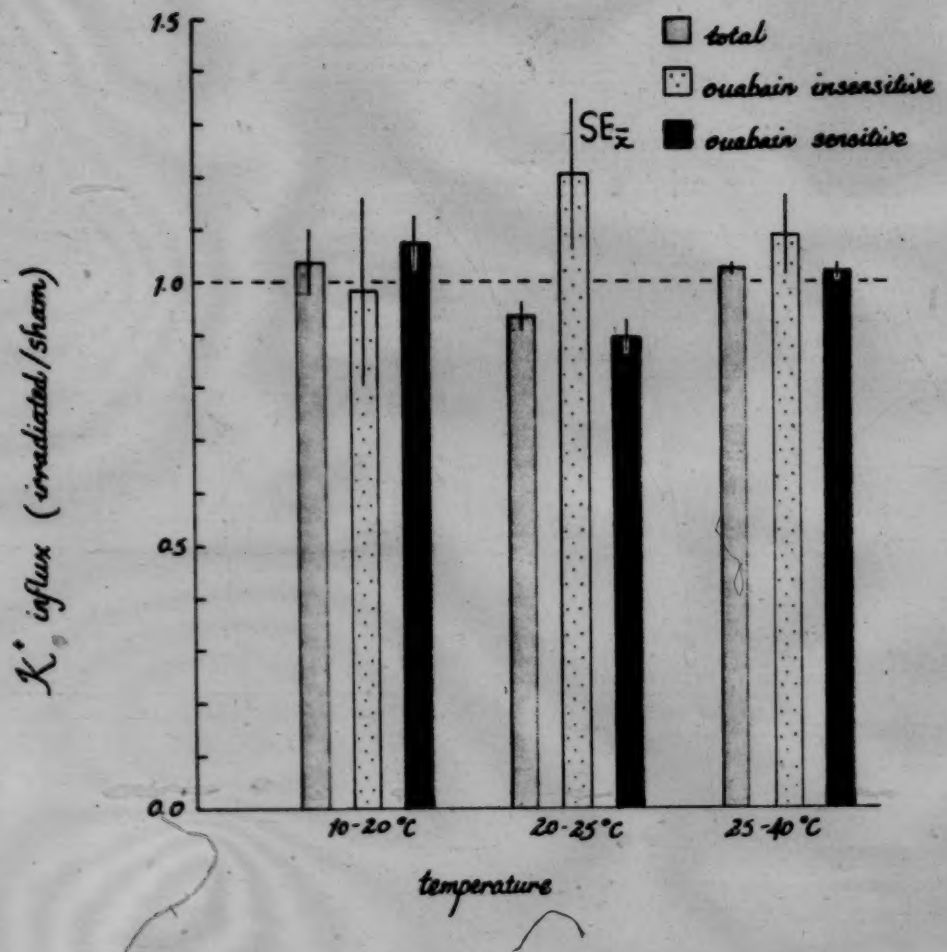


Figure XVIII

The effect of microwaves on K^+ influx in three different incubation temperature ranges. All K^+ influx data were pooled for this comparison.

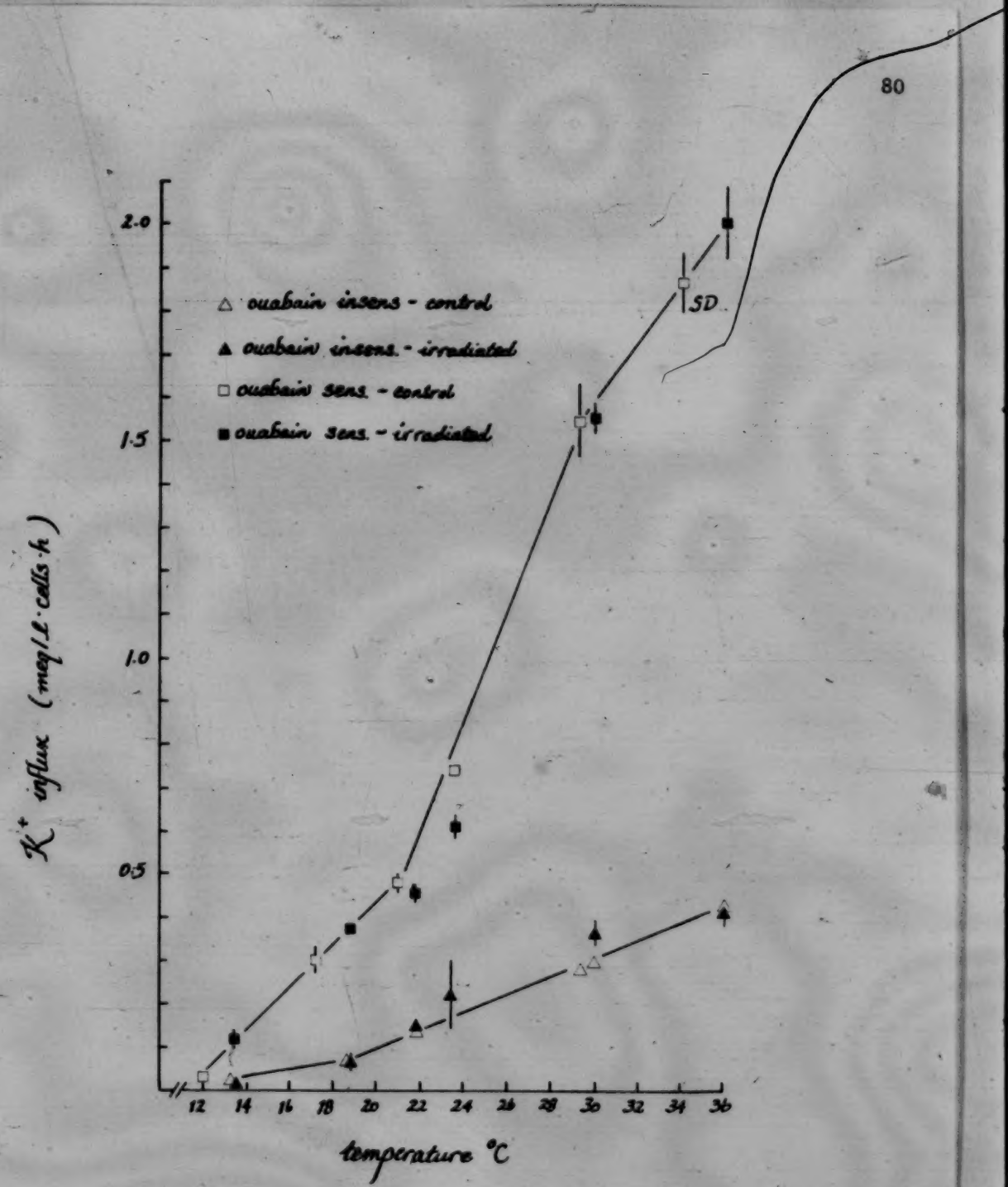


Figure XIX

The effect of microwaves and incubation temperature on the ouabain-sensitive and -insensitive K^+ influx to human erythrocytes.

net decrease in total K^+ influx. In contrast the microwave induced decrease in ouabain-sensitive Na^+ efflux (-0.181 $\pm$ 0.056 meq/ℓ cell·h) was less than the increase in ouabain-insensitive Na^+ efflux (+0.448 $\pm$ 0.114 meq/ℓcell·h)

As with the Na^+ efflux data, the K^+ influx data was grouped according to incubation temperature into three ranges, 10 to 20 °C, 20 to 25 °C and 25 to 40 °C for statistical comparison (Figure XVIII). Within the effective temperature range (20 to 25 °C) there were significant decreases in the total [6.3 ($\pm$ 2.4)%] as well as the ouabain-sensitive [10.6 ($\pm$ 3.4)%] K^+ influxes to irradiated cells. Although there was apparently a 20.5 $\pm$ 14.4% increase in ouabain-insensitive K^+ influx, the variability in the measurements was exceptionally high and precluded a statistically significant change.

The microwave effect on Na^+ efflux and K^+ influx among temperature ranges was compared. The increase in total Na^+ efflux and decrease in total K^+ influx within the 20 to 24.9 °C range were significantly different from those fluxes in the other two ranges, considered independently or in combination (Table 13). The same was true of the increased ouabain-insensitive Na^+ efflux in the effective temperature range (Table 14). There was no significant difference in ouabain-insensitive K^+ influx among temperature ranges, although the data so indicate (Table 14). Even if the latter had been significant, the microwave effect would have been considerably less than that observed on the ouabain-insensitive Na^+ efflux. This finding still supports the possibility that the difference

Table 13

Results of significance testing on the effects of microwaves and incubation temperature on the total flux ratios (irradiated/sham) of Na+ and K+ across the erythrocyte membrane.

		temp. °C	efflux ratio ± SE (n)	t-test between two means: A vs C	A vs B B vs C	A+C vs B
Na+ efflux	A	10-19.9	0.981 ± 0.058 (3)			
					6.988***	
	B	20-24.9	1.231 ± 0.058 (7)	1.475 (ns)		8.873***
					7.324***	
	C	25-40.0	1.040 ± 0.025 (4)			
K+ influx	A	10-19.9	1.040 ± 0.065 (6)			
					3.641*	
	B	20-24.9	0.937 ± 0.024 (6)	0.669 (ns)		6.932***
					7.886***	
	C	25-40.0	1.022 ± 0.011 (6)			

(ns) not significant, P greater than .05
* significant at P less than .05
** significant at P less than .01
*** significant at P less than .001

Table 14

Results of significance testing on the effect of microwaves and incubation temperature on the oubain-insensitive flux ratios (irradiated/sham) of Na+ and K+ across the erythrocyte membrane.

			t-test between two means		
	temp. °C	efflux ratio ± SE (n)	A vs C	A vs B B vs C	A+C vs B
Na+ efflux	A 10-19.9	1.049 ± 0.090(2)			
	B 20-24.9	1.332 ± 0.076(6)	0.621(ns)	3.998* 8.420***	6.303**
	C 25-40.0	1.007 ± 0.049(3)			
K+ influx	A 10-19.9	0.978 ± 0.185(3)			
	B 20-24.9	1.205 ± 0.144(6)	0.943(ns)	1.949(ns) 1.835(ns)	2.372(ns)
	C 25-40.0	1.083 ± 0.076(6)			

(ns) not significant, P greater than .05
* significant at P less than .05
** significant at P less than .01
*** significant at P less than .001

in the direction of the microwave effect on total K^+ influx and Na^+ efflux is primarily due to a difference in the magnitude of the microwave effect on the ouabain-insensitive Na^+ and K^+ fluxes.

The influence of microwaves on ouabain-sensitive Na^+ efflux and K^+ influx within the effective temperature range are complimentary and significant when compared with the fluxes in the adjacent temperature ranges (Table 15). Ouabain-sensitive Na^+ efflux and K^+ influx were depressed by 18.2 ($\pm$ 10.0)% and 10.6 ($\pm$ 3.4)% respectively, in the range of 20 to 24.9 ^oC. Fluxes of control and irradiated samples were compared within each temperature range independent of those fluxes in the other two temperature ranges (Table 16). The effects of microwaves, alluded to above, were shown to be restricted to the 20 to 24.9 ^oC range and were not a result of reversal of the microwave effects between temperature ranges.

The control Na^+ fluxes from cells incubated at 34.7 ^oC were 2.726 $\pm$ 0.005 meq/ℓ cells·h ouabain-sensitive, and 1.455 $\pm$ 0.055 meq/ℓ cell·h ouabain-insensitive (Table 10). These values are comparable to 3.32 and 1.54 meq Na^+/ℓcells·h, respectively, reported for cells previously treated with PCMBS and cysteine, and incubated at 37 ^oC (36). Efflux of Na^+ from these cells are of the same order as the ouabain-sensitive (2.52 - 4.13 meq/ℓ cells·h), ouabain-insensitive (0.94 - 1.79 meq/ℓ cells·h) and total (2.52 - 4.50 meq/ℓcells·h) Na^+ effluxes from untreated erythrocytes (24, 37, 50, 74, 106). The above comparison establishes that the decreased Na^+ efflux from 0.1

Table 15

Results of significance testing on the effects of microwaves and incubation temperature on the ouabain-sensitive flux ratios (irradiated/sham) of Na+ and K+ across the erythrocyte membrane.

		temp. °C	efflux ratio ± SE (n)	t-test between two means: A vs C	A vs B / B vs C	A+C vs B
Na+ efflux	A	10-19.9	1.050 ± 0.127(2)	0.629(ns)	2.352(ns) / 6.325**	5.813**
	B	20-24.9	0.818 ± 0.100(6)			
	C	25-40.0	1.108 ± 0.036(3)			
K+ influx	A	10-19.9	1.075 ± 0.056(6)	2.239(ns)	6.767** / 7.621***	9.399***
	B	20-24.5	0.894 ± 0.034(6)			
	C	25-40.0	1.020 ± 0.022(6)			

(ns) not significant, P greater than .05
* significant at P less than .05
** significant at P less than .01
*** significant at P less than .001

Table 16

The effect of microwaves on Na+ and K+ fluxes across the erythrocyte membrane at different incubation temperatures. D(meq/ℓ cell·h) = irradiated cell flux - control cell flux

	Temp °C	D ± S_D(n)	t	P<
TOTAL				
----------	10-20	+0.004±0.047(4)	0.085	(ns)
Na+ EFFLUX	20-25	+0.457±0.115(7)	3.974	.01
----------	25-40	+0.114±0.064(6)	2.250	(ns)
----------	10-20	0.000±0.009(6)	0.000	(ns)
K+ INFLUX	20-25	-0.052±0.021(6)	2.515	(ns)
----------	25-40	+0.040±0.027(6)	1.503	(ns)
OUABAIN INSENSITIVE				
----------	10-20	+0.029±0.076(2)	0.382	(ns)
Na+ EFFLUX	20-25	+0.448±0.114(6)	3.930	.05
----------	25-40	+0.014±0.059(4)	0.237	(ns)
----------	10-20	+0.006±0.006(6)	1.000	(ns)
K+ INFLUX	20-25	+0.032±0.024(6)	1.337	(ns)
----------	25-40	+0.019±0.025(6)	0.757	(ns)
OUABAIN SENSITIVE				
----------	10-20	+0.002±0.024(2)	0.083	(ns)
Na+ EFFLUX	20-24	-0.181±0.056(5)	3.236	.01
----------	24-40	+0.161±0.058(4)	2.767	(ns)
----------	10-20	+0.010±0.007(6)	1.490	(ns)
K+ INFLUX	20-24	-0.074±0.027(6)	2.763	.01
----------	24-40	+0.044±0.043(6)	1.023	(ns)

(ns) - not significant

mM PCMBS treated cells was completely restored by incubation with 2 mM cysteine. This agrees with the report that inhibition of $(Na^+ + K^+)$ATPase activity in PCMBS treated erythrocytes is completely reversed by cysteine (104). Furthermore, the level of bound Hg^{++}, determined by neutron activation analysis (NAA), was reduced from 4.5×10^{-5} mole/cell during $^{24}Na^+$ loading (116) to 3.73×10^{-20} mole/cell [0.0016% of PCMBS titratable -SH groups (36)] after cysteine washing. Osmotic fragility, a sensitive indicator of Hg^{++} bound to erythrocytes, is unaffected at levels lower than 10^{-18} mole Hg^{++}/cell (116).

The total K^+ influx for control cell suspensions incubated at 34.1 °C was 2.304 ± 0.019 meq/ℓ cells·h (Table 12). This value is comparable to the total K^+ influx to cells incubated at 29 °C: 1.50 ± 0.04 meq/ℓ cells·h (37), and at 37.7 °C: 3.40 ± 0.66 meq/ℓ cells·h (74). At 34.1 °, 73% of the total K^+ influx to control cells was ouabain sensitive, consistent with the 60 to 70% reported by others for cells incubated at 37 °C (74).

Stoicheometry of 2-3Na^+ : 2K^+ transported per cycle of the $(Na^+ + K^+)$ATPase is now generally accepted (89, 92). This value is based on experimentally derived ratios of 2.27 - 2.82 Na^+ : 2 K^+ (34, 74, 106) and agrees with 2.15 (± 0.63) Na^+ : 2K^+ derived from the data reported here. Some flexibility in the Na^+ : K^+ transport stoicheometry has been acknowledged (89).

Ouabain-sensitive Na^+ and K^+ fluxes in human (106) and canine (26) erythrocytes are temperature-sensitive. However,

different proportions of ouabain-sensitive and -insensitive Na^+ and K^+ fluxes were observed at different temperatures (Table 10, Table 12). There is some support for this observation; Joyce and Weatherall (59) reported that 88% of total K^+ influx is inhibited by digoxin at 37 ^oC but only 55% at 7 ^oC, and Glynn (37) derived different Q_{10} values for external K^+-dependent Na^+ efflux. The differences observed in the temperature dependence of the ouabain-sensitive and -insenstive Na^+ and K^+ fluxes concur with these reports by virtue of their dependence on external K^+ concentration (117).

The synergism indicated in the ANOVA (Table 11) supports the suggestion that microwaves influence Na^+ efflux in at least two ways:

1) by affecting the ouabain-sensitive, $(Na^+ + K^+)$ATPase Na^+ efflux vector, and,

2) by affecting some part of the ouabain-insensitive Na^+ efflux.

Very little is known about the ouabain-insensitive Na^+ and K^+ transport pathways in human erythrocytes except that at least a portion of each is by diffusion directly across the lipid bilayer. The microwave-induced increase in ouabain-insensitive Na^+ efflux is, consequently, cause only for speculation. Microwaves may influence simple Na^+ diffusion across the bilayer but since microwaves do not affect Na^+ leakage from purely lipid vesicles this would have to be associated with the protein component of the membrane. Alternately microwaves may increase the rate of

ouabain-insensitive exchange diffusion (8, 50, 74, 117) or the rate of the hypothetical, ouabain-insensitive Na^+ pump (50).

These observations agree with those of Ismailov (53, 54) who reported different microwave effects on Na^+ efflux from erythrocytes incubated with and without mono-iodoacetate, an inhibitor of $(Na^+ + K^+)$ATPase (51). The differences revealed inhibition of iodoacetate-sensitive and stimulation of iodoacetate-insensitive Na^+ efflux from human erythrocytes incubated at 37 ^oC and exposed to 1009-MHz waves. Although no temperature specificity for these effects was reported, the frequency difference may account for the absence of this effect at physiological temperatures in our experiments (Table 10, Figure XVII).

Microwave effects on Na^+ efflux occuring only at specific temperatures have been previously reported. Olcerst et al (81) observed increased $^{22}Na^+$ efflux from ouabain treated rabbit erythrocytes exposed to 2450-MHz but only in the range of 8 to 13 ^oC and at 22.5 ^oC and 38 ^oC. The effective temperatures reported by Olcerst et al were clearly correlated with discontinuities in an Arrhenius plot of Na^+ efflux from ouabain-treated rabbit red cells. No such discontinuity was observed in the Arrhenius plot of Na^+ efflux from human erythrocytes within the 20 to 25 ^oC range (Figure XX). Discounting species differences, the possibility remains that some event may occur at a specific temperature within human erythrocyte membranes making them sensitive to microwaves. This event may not be manifested as a discontinuity in the Arrhenius

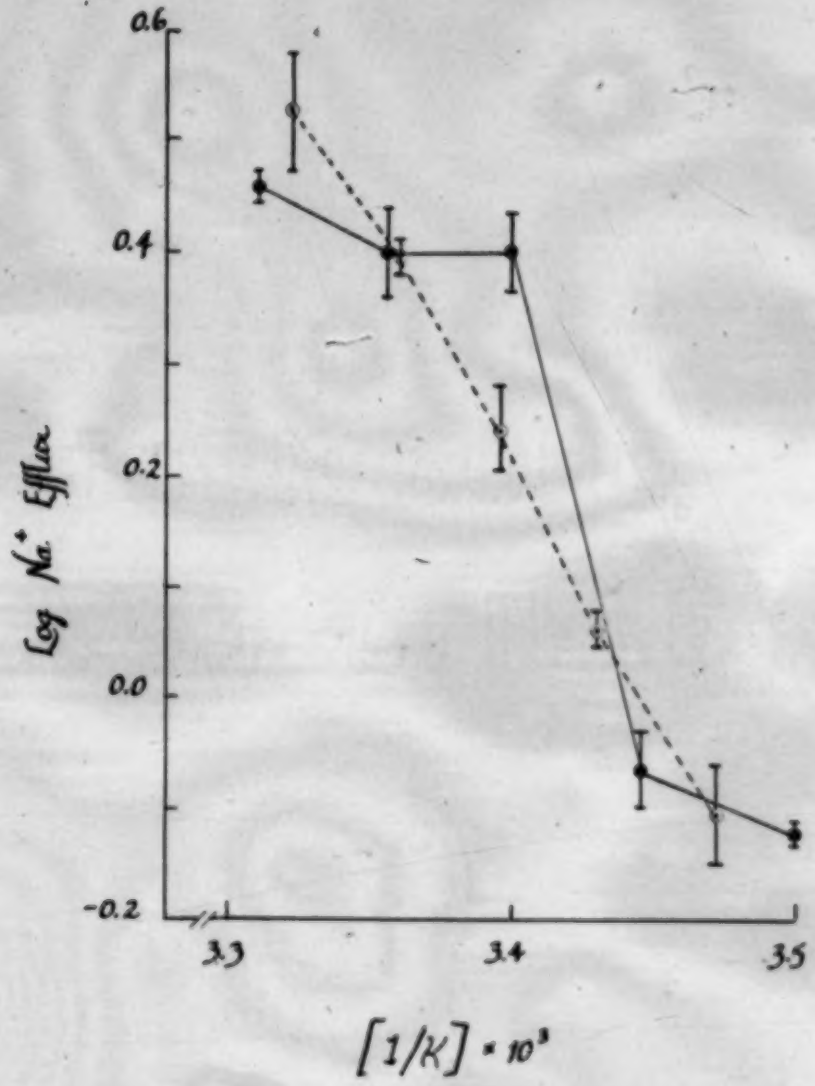


Figure XX

The effect of microwaves and incubation temperature on total Na^+ efflux from the human erythrocytes of a single donor. Results are shown as an Arrhenius plot. The activation energy of the Na^+ efflux is 17099 ± 7181 cal/mole as derived from regression analysis of the control data.

plot.

The data presented so far indicates that:

1) The effect of microwaves on Na^+ and K^+ fluxes is temperature sensitive: restricted to the 20 to 24.9 ^oC range.

2) The ouabain sensitive Na^+ efflux and K^+ influx found to be affected by microwaves in the same direction and magnitude indicates that (Na^++K^+)ATPase function is somehow depressed by microwaves.

3) The microwave effect on ouabain-insensitive fluxes of Na^+ and K^+ differ in magnitude but must both be mediated by microwaves interacting with protein or lipid-protein complexes since microwaves did not affect Na^+ self-diffusion across lipid bilayers.

The effect of microwaves on ouabain-insensitive Na^+ and K^+ fluxes is extremely difficult to interpret. Analysis of the linear components of Na^+ efflux and K^+ influx have been reported to correspond to permeabilities of 10^{-10} cm/s and 1.1 to 2.4 x 10^{-10} cm/s respectively (8, 57), values that are comparable to those reported for lipid bilayers (65). Based on ouabain-insensitive cation fluxes from control cells (Table 10, Table 12), P_{Na^+} of the erythrocyte membrane was calculated to be 18.9 x 10^{-10} cm/s and P_{K^+} as 7.09 x 10^{-10} cm/s at 24 ^oC. The inflated permeabilities may result from Na^+/Na^+ and K^+/K^+ exchange in the ouabain-poisoned cells (8). The higher level of Na^+ permeation compared with that of K^+, may account for the more pronounced influence of microwaves on Na^+ efflux in the presence of ouabain. Dunn (25) and others (50,74) have provided evidence for Na^+ exchange-diffusion not inhibited by

ouabain. In the presence of ethacrynic acid, a purported inhibitor of the ouabain-insensitive Na^+ exchange diffusion (50, 74), the level of Na^+ efflux falls to about 0.5 meq/ℓ cells·h (25). This value of Na^+ efflux compared with the level of ouabain-insensitive K^+ influx is more a realistic reflection of P_{Na^+} compared to P_{K^+}. The ouabain-insensitive Na^+ exchange diffusion has been attributed to another Na^+ pump in the red-cell membrane (50). It has been speculated that ouabain-insensitive Na^+ efflux may be another transport mode of the $(Na^+ + K^+)$ATPase (25, 117). The interaction of the $(Na^+ + K^+)$ATPase that results in the inhibition of ouabain-sensitive Na^+ and K^+ transport may also be responsible for the increased ouabain insensitive Na^+ efflux.

V - NET FLUX EXPERIMENTS

1 : INTRODUCTION

The experiments discussed so far deal only with unidirectional fluxes of Na^+ and K^+. These cations move in both directions across the membrane, however, and it is the net fluxes of Na^+ and K^+ that are of consequence to the function of the erythrocyte. Under normal physiological conditions the net flux of both cations is zero. The active efflux of Na^+ and influx of K^+ is balanced by leakage of the ions down their respective concentration gradients and by co-transport processes with other substrates (97, 119). Thus, concentrations of Na^+ and K^+ on either side of the membrane remain constant. To assess the effect of low-level microwave radiation on net cation flux, the intra- and extracellular concentrations of Na^+ and K^+ were measured in suspensions of intact erythrocytes before and after irradiation.

2 : MATERIALS AND METHODS

Venous blood from a human volunteer was drawn prior to the experiment. The blood was centrifuged at 2300 x g for 5 min, and the cell pellet was resuspended in 20 vol buffer containing 2.5 mM Na_2HPO_4, 145 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$ and 11 mM Dextrose at pH = 7.4, and recentrifuged. The cells were washed in this way three times and finally was resuspended to a hematocrit

of 10%. Aliquots (1.5 ml) of this suspension were incubated with and without 10^{-4} M ouabain at predetermined temperatures under control and irradiation conditions in exposure chamber #4.

After incubation each aliquot was divided into 3 replicate samples layered on top of 0.2 ml of dibutyl phthalate in 1.5-ml Eppendorf centrifuge tubes. The replicates were centrifuged at 2500 x g for 1 min and the supernatant removed for extracellular Na^+ and K^+ determination. The dibutyl phthalate layer was removed, and the cells were lysed in an equal volume of distilled water before determination of cellular Na^+ and K^+ by flame photometry (Corning, Model 430).

3 : RESULTS AND DISCUSSION

Erythrocytes exposed to low level microwaves exhibit enhanced Na^+ efflux both in the absence (+0.457 meq/ℓ cell·h) and presence (+0.448±0.114 meq/ℓ cell·h) of ouabain (Table 16). Meanwhile, K^+ influx decreases nominally (-0.052 meq/ℓ cell·h) in the absence of ouabain (Table 16). Discounting any backflux of either cation, it may be anticipated that during 2-h periods of irradiation these effects would result in significant decreases in cell Na^+ (1 to 2 meq/ℓcells) with and without ouabain. However, exposure to low-level 2450-MHz microwaves did not significantly affect the distribution of Na^+ (Table 17) or K^+ (Table 18) across the erythrocyte membrane but the results indicate that in the absence of ouabain there is an accumulation of Na^+ by exposed erythrocytes incubated in the 20 to 25 ^oC range.

Table 17

The effect of low-level microwave radiation, for 2 h, on the intra- and extracellular concentrations of Na^+ in a suspension of intact erythrocytes (hct = 10%) incubated with (10^{-10} M) and without ouabain.

			[Na] (meq/l)			
Ouabain	Time h	Temp °C	IRRADIATED		IRRADIATED	
			Cell	Buffer	Cell	Buffer
-	0		12±1.5	148±0.8	12±1.5	148±0.8
-	2	37	15±1.8	148±0.8	15±1.8	148±0.8
-	2	30	14±1.7	148±0.8	18±2.2	148±0.8
-	2	25	14±1.7	148±0.8	20±2.5	148±0.8
-	2	22	15±1.8	148±0.8	20±2.5	148±0.8
-	2	21	15±1.8	147±0.8	18±2.2	148±0.8
-	2	20	15±1.8	149±0.8	15±1.8	148±0.8
-	2	17	15±1.8	148±0.8	15±1.8	148±0.8
-	2	10	15±1.8	148±0.8	15±1.8	148±0.8
+	0		12±1.5	149±0.8	12±1.5	149±0.8
+	2	37	18±2.2	149±0.8	14±1.7	148±0.8
+	2	30	15±1.8	148±0.8	13±1.6	148±0.8
+	2	25	15±1.8	148±0.8	15±1.8	148±0.8
+	2	22	15±1.8	148±0.8	15±1.8	148±0.8
+	2	21	18±2.2	147±0.8	18±2.2	148±0.8
+	2	20	15±1.8	147±0.8	15±1.8	146±0.8
+	2	17	15±1.8	148±0.8	15±1.8	148±0.8
+	2	10	15±1.8	147±0.8	15±1.8	148±0.8

Table 18

The effect of low-level microwave irradiation, for 2 hr, on the intra- and extracellular concentrations of K^+ in a suspension of intact erythrocytes (hct = 10%) incubated with (10^{-10} M) and without ouabain.

Ouabain	Time h	Temp °C	$[K^+]$ (meq/l) IRRAD cell	IRRAD buffer	cell	buffer
-	0	23	96±4	4.5±0.1	96±4	4.5±0.1
-	2	23	95±2	4.3±0.1	97±2	4.3±0.1
-	4.5	23	88±2	4.7±0.1	88±2	4.7±0.1
-	20	4	79±2	5.1±0.1		
+	0	23	96±4	4.5±0.1	96±4	4.6±0.1
+	2	23	96±2	4.4±0.1	89±2	4.4±0.1
+	4.5	23	91±2	4.9±0.1	91±2	4.9±0.1
+	20	4	80±2	5.1±0.1		

The changes in K^+ influx which occured during irradiation of the cells, although significant, are small [-0.074 to +0.032 meq/ℓcell·h (Table 16)] and within the error limits reported in Table 18 (0.1 to 4.0 meq/ℓ) for intra and extracellular K^+ concentrations. It is not surprising that these differences in flux rate were not manifested as K^+ concentration changes across the cell membrane. The larger changes reported in total Na^+ efflux within the effective temperature range could have caused a noticable decrease in cellular Na^+. However, the results suggest a net accumulation of Na^+ by irradiated cells (Table 17). Observations by Ismailov (53) support the trends reported here. He observed that the rate of accumulation of Na^+ by intact but irradiated cells was more than twice the rate of loss

of K^+ by the same cells. Considering only the microwave induced decrease in ouabain-sensitive Na^+ efflux, this result would have been expected. The rate of backflux into the cell would have exceeded the depressed active efflux of Na^+. One possible explanation is that transmembrane diffusion of Na^+ is increased in both directions by microwaves, as suggested by the increased ouabain insensitive Na^+ efflux. Therefore, of the reported microwave effects on Na^+ efflux only the decrease in ouabain sensitive Na^+ efflux would affect cellular Na^+ concentration. The observation that there was no apparent net accumulation of Na^+ by microwave exposed cells incubated with ouabain supports this conclusion.

Like all transport functions the levels of both passive and active Na^+ and K^+ fluxes depend on the concentrations of these cations across the membrane (37, 106). Flame photometry is not as sensitive a technique as the radiotracer techniques used to follow cation movements in the erythrocyte. However, any changes in cell Na^+ or K^+ sufficient to cause the magnitude of observed effects on the fluxes of these cations would surely have been detected. The demonstrated stability of cellular and extracellular Na^+ and K^+ concentrations indicates that the changes in the ouabain-sensitive and -insensitive Na^+ and K^+ fluxes are due to some direct influence of microwaves on Na^+ and K^+ transport mechanisms and are not secondary to some previously undetected change in the Na^+ K^+ gradient across the erythrocyte membrane.

VI - ATPase EXPERIMENTS

1 : INTRODUCTION

The $(Na^+ + K^+)$ATPase is the membrane protein responsible for the active translocation of Na^+ and K^+ in a number of membrane types (24, 92, 98, 103, 117). However, the exact mechanism whereby the splitting of the ATP results in cation translocation remains unclear (68, 89, 92, 98). Considering the observed effects of microwaves on the ouabain-sensitive fluxes of Na^+ and K^+, the following experiments were designed to probe the influence of microwaves on the ATPase activity in erythroctes specifically that of the $(Na^+ + K^+)$ATPase.

Since the active site of the ATPase is at the inner surface of the membrane, the ATPase activity of microwave-exposed fragmented erythrocyte ghosts, rather than that of intact erythrocytes, was tested. The ATPase thus had access to ATP in solution and, even in the absence of Na^+ and K^+ gradients, erythrocyte ghosts have been shown to be a reliable test system (24, 100).

The level of $(Na^+ + K^+)$ATPase activity in human erythrocytes is relatively low (~0.03 μmol Pi/mg protein·h) compared with other tissues (98); it can still be accurately resolved beyond the background (Mg^+)ATPase (~1 μmol Pi/mg protein·h) by omitting Na^+ or K^+, the coactivators of the

$(Na^+ + K^+)$ATPase, from control solutions (100). The $(Na^+ + K^+)$ATPase activity of erythrocyte ghosts exposed to low-level microwaves, at several discrete incubation temperatures over the 10 to 40 ^oC range, was observed. Within the 20 to 25 ^oC range $(Na^+ + K^+)$ATPase activity was depressed by microwaves at a magnitude that complimented the observed effects of microwaves on ouabain-sensitive Na^+ and K^+ fluxes.

2 : MATERIALS AND METHODS

2-1 : Preparation of Erythrocyte Ghosts

Packed human red-blood cells, never more than one month old, were provided by the University of Alberta Hospital Blood Bank. Erythrocyte ghosts were prepared by the method of Dodge et al (22). The erythrocytes were suspended in 3 vol of buffer containing 150mM NaCl and 5mM NaH_2PO_4 at pH = 8 and separated by centrifugation at 2500 x g. This procedure was repeated twice. The packed cells were then hemolysed in 40 vol of 5mM NaH_2PO_4 at pH= 8 and allowed to stand for 30 min. The fragmented ghosts were concentrated by centrifugation at 22000 x g for 15 min. The pellet was washed and recentrifuged, as many times as needed, to clear the supernatant of hemoglobin. After standing overnight in 3 vol of lysing buffer at 4 ^oC, the ghosts were washed twice, clearing the ghosts of any remaining colour. The pellets were then frozen and thawed to break any ghosts that may have resealed (24). The ghosts were stored frozen (-20 ^oC) until needed. No loss of ATPase activity was noted over a two months period.

2-2:Experimental Procedure

Packed ghosts were suspended in a buffer containing 30mM Tris-HCl, 100mM NaCl, 10mM KCl, 5mM $MgCl_2$, 1mM cysteine and 5mM ATP adjusted to pH = 7, or in a similar buffer containing 110mM NaCl but no KCl. The difference in the ATPase activity of these two mixtures is an index of the $(Na^+ + K^+)$ATPase activity (100).

For each experiment two 0.9-ml samples-- one with and one without K^+-- were placed in sample chamber #4 (Fig. VII) in the waveguide system; an identical pair was placed in the control-waveguide section. The samples were irradiated for 3 h after which the temperature of each sample was measured by a microthermocouple. The ATPase reaction was quenched by adding 0.1 ml 55M trichloroacetic acid, and the samples were assayed for phosphorus by the Fiske-Subbarow method (32). Phosphatase activity was determined as μ mol Pi evolved per mg total ghost protein assayed by the Lowry method (73), which was modified by the addition of deoxycholate. The quantity of ghost protein per litre of packed whole cells was also determined by the latter method.

3 : RESULTS AND DISCUSSION

Low-level 2450-MHz microwaves (SAR = 2 to 3 mW/g) caused a 2.8 ($\pm$0.4)% increase in K^+-independent and a 35.6 ($\pm$4.4)% decrease in K^+-dependent ATPase activity in human erythrocyte ghost suspensions incubated at temperatures in the 21 to 25 ^{o}C range (Figure XXII). There were no microwave effects resolved by comparing total ATPase activity in control and exposed ghost

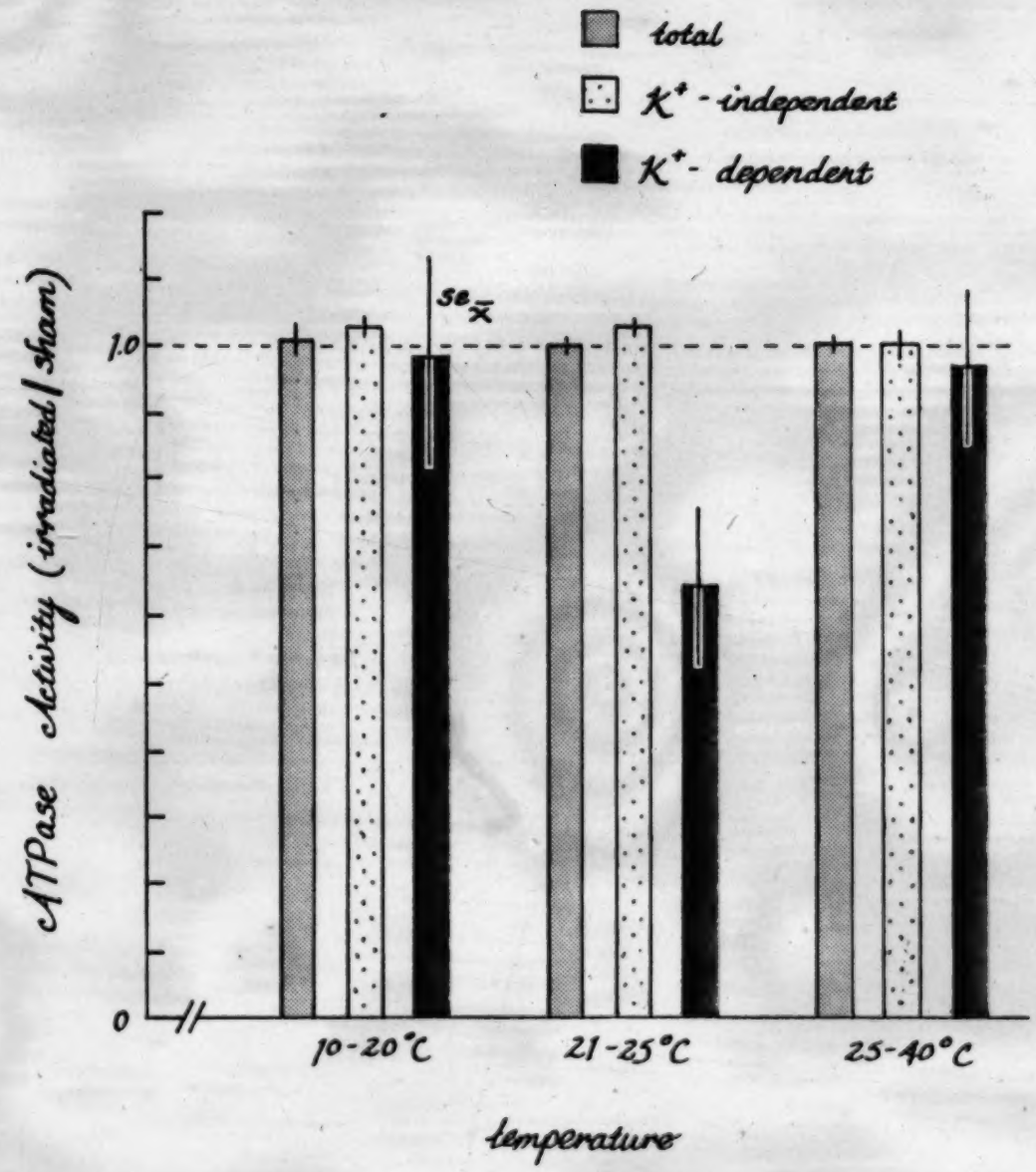


Figure XXI

The effect of microwaves on ATPase activity in erythrocyte ghosts incubated in three different temperature ranges.

suspensions within the temperature range tested (Table 19).

Table 19

The effects of microwaves and incubation temperature on the ATPase activity (irradiated/sham) in human erythrocyte ghosts.

Temp^{o}	ATPase activity (irradiated/sham) ± SE(n)		
	total	K-independent	K-dependent
10 - 21	1.007±0.012(4)	1.082±0.008(4)	0.985±0.080(4)
21 - 24	0.997±0.005(7)	1.028±0.004(7)	0.644±0.044(7)
24 - 40	0.999±0.002(8)	1.005±0.007(8)	0.968±0.041(8)

The observed level of $(Na^{+}+K^{+})$ATPase activity is comparable to that previously reported for erythrocyte ghost preparations. At 38 ^{o}C, the ghosts exhibited 0.033±0.003μmol P_i/mg protein·h, a value within the range described by Post et al (88): 0.041±0.020 μ mol P_i/mg protein·h. To relate the results of the ATPase experiments to those of the flux experiments, all values of ATPase activity were converted from μ mol P_i/mg ghost protein·h to mmol P_i/ℓ cells·h. The ghosts were found to contain 1.805±0.048 g ghost protein per litre of the originaly packed erythrocytes. This value is comparable but lower than 7.597 g protein/litre original packed cells, derived from published data (41). One must acknowledge some variability in

quantity of ghost protein as a result of the severity and extent of different preparation procedures for erythrocyte ghosts (88). The value, 1.805 g ghost protein/litre packed cells was applied to the data for the initial unit conversion: .space0

$$\frac{1.805 \text{ g protein}}{\ell \text{ cells}} \times \frac{\mu\text{mol } P_i}{\text{mg protein} \cdot \text{h}} = \text{ATPase activity } \frac{\text{mmol } P_i}{\ell \text{cell} \cdot \text{h}}$$

The resulting ATPase activity levels (for example 0.060 mmol P_i/ℓcell·hr at 38 ^{o}C) were found to be 5 to 10% of that reported for whole erythrocytes (1.12±0.02 mmol/ℓ cells·h at 37 ^{o}) (11).

Post et al (88) have reported that only 8% and 22% of the original K^+-dependent and K^+-independent ATPase activities, respectively, are recovered in hemoglobin free ghosts. The K^+-dependent and K^+-independent ATPase levels were corrected according to these proportions. The corrected values of $(Na^+ + K^+)$ATPase activity (Table 20) were of the same magnitude as those reported for intact human erythrocytes (11). The K^+-dependent ATPase activity is assumed to be that portion catalysed by the $(Na^+ + K^+)$ATPase because of the absolute dependence on both cations. The level of ATPase activity in human erythrocytes has been shown to be the same in the absence of K^+ and in the presence of ouabain (24, 88). Therefore, the microwave induced decrease in the K^+-dependent ATPase activity represents an effect on the $(Na^+ + K^+)$ATPase activity.

Table 20

The effect of low level 2450 MHz microwaves on the K^+ independent and K^+-dependent ATPase activity in a suspension of fragmented human erythrocyte ghosts incubated at different temperatures.

Temp. C	ATPase activity (mmol P_i/ℓ cells·hr ± SEM)			
	K^+ - independent		K^+ - dependent	
	sham	irrad	sham	irrad
11.8	0.287a	0.300±0.037	0.305a	0.416±0.045
12.3	0.378±0.074		0.282±0.068	
15.9	0.817a	0.841±0.025	0.361a	0.327±0.068
16.1	0.841a	0.866±0.049	0.361a	0.305±0.068
16.4	0.915±0.013		0.316±0.034	
16.6	0.886±0.025		0.395±0.034	
20.4	1.493±0.021		0.452±0.043	
20.6	1.535a	1.522±0.017	0.429a	0.519±0.023
20.8	1.600±0.029		0.452±0.045	
21.9	1.850a	1.883±0.037	0.452a	0.271±0.034
22.2	1.924a	1.965±0.013	0.463a	0.361±0.000
22.4	1.969±0.013	2.027±0.008	0.418±0.012	0.339±0.045
22.9	2.101a	2.166±0.037	0.463a	0.282±0.079
23.2	2.187±0.025		0.406±0.045	
23.4	2.215a	2.326±0.022	0.474a	0.282±0.023
23.5	2.228±0.037		0.553±0.068	
23.7	2.289a	2.363±0.017	0.474a	0.215±0.034
23.9	2.322±0.013		0.530±0.068	
24.1	2.388a	2.433±0.025	0.485a	0.350±0.068
24.6	2.482±0.037		0.598±0.091	
24.8	2.560±0.021		0.485±0.147	
25.3	2.769a	2.691±0.013	0.508a	0.440±0.023
26.5	2.987±0.000		0.519±0.023	
26.7	3.020±0.004		0.530±0.192	
26.9	3.068a	3.023±0.025	0.530a	0.632±0.023
27.9	3.315a	3.409±0.029	0.508a	0.530±0.045
30.6	4.029a	4.000±0.013	0.576a	0.508±0.057
31.4	4.090±0.008		0.497±0.034	
32.4	4.176a	4.148±0.029	0.609a	0.553±0.124
32.9	4.226±0.123		0.621±0.226	
34.1	4.312a	4.295±0.021	0.632a	0.542±0.045
34.6	4.361±0.066		0.779±0.215	
35.9	4.472a	4.656±0.078	0.666a	0.650±0.124
36.9	4.537±0.062		0.576±0.034	
38.8	4.693a	4.660±0.037	0.699a	0.734±0.034

a - values estimated from regression analysis of control data

The 0.165±0.023 mmol ATP/ℓ cells·h decrease in the $(Na^+ + K^+)$ATPase activity (Table 21) compliments the decreases observed in ouabain-sensitive Na^+ and K^+ fluxes in exposed erythrocytes. It is safe to assume that these manifestations in exposed erythrocytes result from the same microwave effect since the active transport of Na^+ and K^+ is driven by the $(Na^+ + K^+)$ATPase. Furthermore, the microwave effect on the $(Na^+ + K^+)$ATPase is exclusively restricted to the same temperature range (Figure XXII) in which the cation flux effects are apparent.

Table 21

The effect of microwaves on ATPase activity in erythrocyte ghosts at different incubation temperatures. D is the mean difference (sham-irradiated) in ATPase activity (mmol ATP/l-cells·hr).

	Temp°C	$D \pm S_D$ (n)	t	P<
----	10-21	+0.012±0.008 (4)	1.921	(NS)
K-INDEP	21-24	+0.062±0.008 (7)	5.990	.001
----	24-40	+0.020±0.029 (8)	0.695	(NS)
----	10-21	+0.004±0.034 (4)	0.115	(NS)
K DEP	21-24	-0.165±0.023 (7)	7.768	.001
----	24-40	-0.026±0.019 (8)	1.400	(NS)

(NS) - not significant .05<P

The increase in K^+-independent ATPase activity may correlate with the increased ouabain-insensitive Na^+ efflux

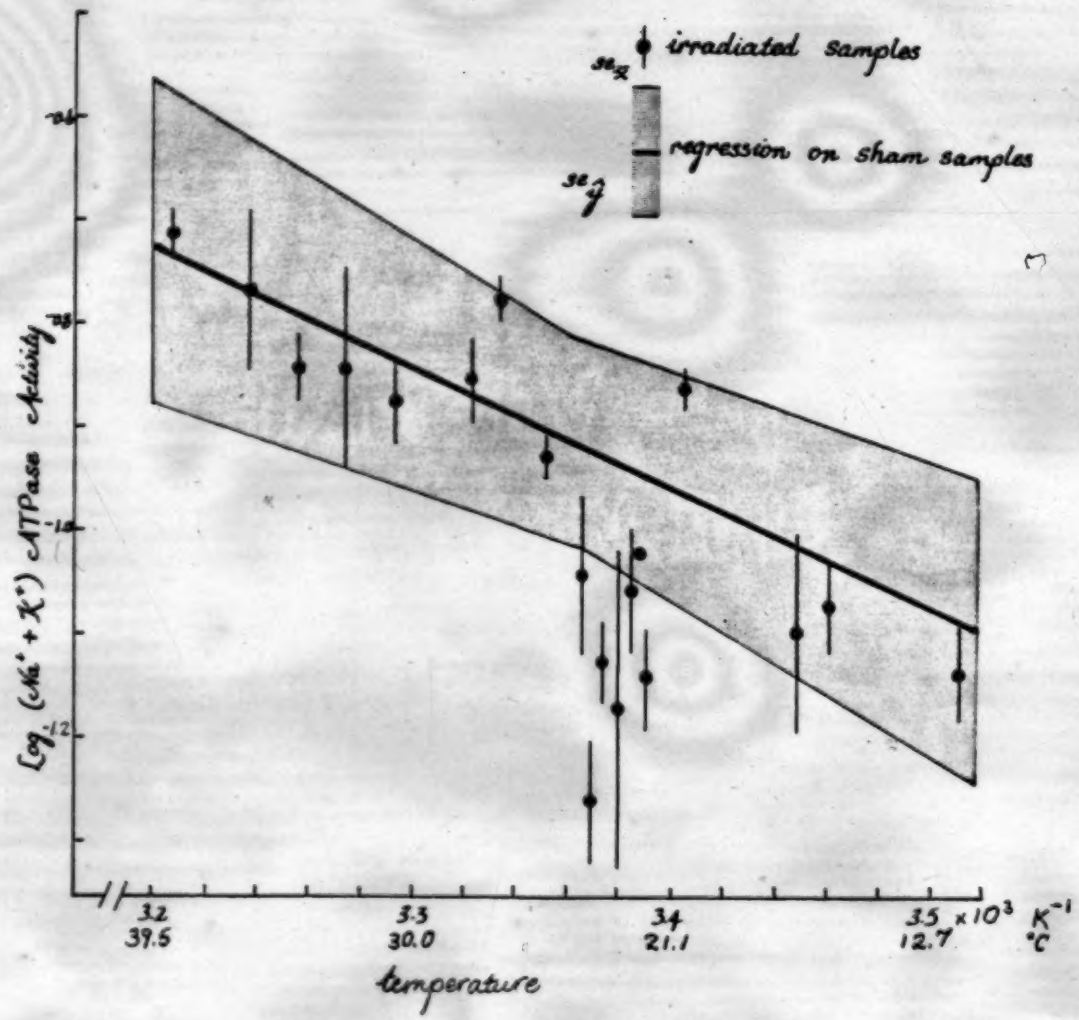


Figure XXII

The effect of microwaves and incubation temperature on the K^+-dependent or $(Na^+ + K^+)$ATPase activity of erythrocyte ghosts.

observed in microwave-exposed erythrocytes. The hypothetical, ouabain-insensitive Na^+ pump, alluded to previously, has been proposed to be an ATPase akin to the $(Na^+ + K^+)$ATPase but which uses a counter-ion other than K^+ (50). Considering the potential similarities between these enzymes, it is possible that microwaves affect the interaction between the transport enzyme and ATP in both systems.

Previous reports of microwave effects on ATPase activity are few, conflicting and indirect. ATP levels have been shown to fall more slowly in excised rat brains exposed to 591-MHz radiation (13.8 mW/cm^2) than in control brains (95). The magnitude of the effect was temperature dependent. In contrast, 2350-MHz microwaves did not significantly affect total ATPase activity in erythrocytes incubated at 38 ^oC (2). The latter finding does not conflict with the results of this work: although microwaves caused a decrease in $(Na^+ + K^+)$ATPase activity, no significant effect was observed on total ATPase activity of the erythrocytes at any temperature (Table 19).

Another report provides convincing but indirect evidence of a low-level 2450-MHz microwave effect on $(Na^+ + K^+)$ATPase. McRee and Wachtel (79) observed that repeatedly stimulated frog sciatic nerves lost vitality in shorter periods of time when exposed to 2450 MHz radiation (SAR=10 mW/mg) than did control nerves. The microwave effect on nerve vitality was no longer apparent in nerves pretreated with ouabain, a specific inhibitor of the $(Na^+ + K^+)$ATPase. The effect was attributed to the accelerated loss of Na^+ and K^+ gradients, due to microwave

inhibition of the $(Na^+ + K^+)$ATPase.

VII - DISCUSSION

Since microwaves did not affect leakage from egg-PC or RBC-lipid vesicles, all effects on Na^+ and K^+ transport in irradiated human erythrocytes must ultimately involve an interaction between the microwaves and membrane proteins or lipid/protein complexes. It also appears that at least two transport vectors for these cations are differentially affected, and that the effects only manifest themselves in the temperature range 20 to 25 °C. First, (Na^++K^+)ATPase mediated transport of Na^+ and K^+ was inhibited by 18.2 (±10.0)% and 10.6 (±3.4)% respectively (Table 16) in the presence of microwaves. Observed decreases in ouabain-sensitive fluxes of Na^+ (-0.181±0.056 meq/ℓ cell·h) and K^+ (-0.074±0.027 meq/ℓ cell·h) and in K^+-dependent ATPase activity (-0.165±0.023 mmol P_i/ℓcell·hr) in irradiated erythrocytes incubated in the effective temperature range, support this conclusion. Second, low-level irradiation increased ouabain insensitive Na^+ efflux from erythrocytes at temperatures between 20 and 25 °C through some, as yet undetermined, pathway.

The magnitude of the latter effect, an increase of 0.448±0.114 meq/ℓcells·h, is not paralleled by the much smaller increase in ouabain-insensitive K^+ influx (0.032±0.024 meq/ℓcells·h) in irradiated cells. The lack of a significant microwave effect on intracellular Na^+ concentration indicates that a large part of the microwave-induced

increase of Na^+ efflux may be due to an increase in a ouabain-insensitive Na^+/Na^+ exchange diffusion (25, 74, 117). Conversly, this effect could present itself as the result of a microwave-induced increase in the hypothetical, ouabain-insensitive Na^+ pump (50), and a simultaneous increase in Na^+ influx, due to an induced increase in P_{Na^+}, to accomodate the lack of a microwave effect on net Na^+ flux. In normal erythrocytes P_{K+} is 2 to 3 fold higher than P_{Na^+} (57). There was only a small increase (+0.032 meq/ℓ cell·h) in ouabain-insensitive K^+ influx. If the simple Na^+ diffusion had increased to the same degree during microwave irradiation it could not have compensated for the larger (+0.448 meq/ℓcell·h) increase in ouabain-insensitive Na^+ efflux and a net Na^+ efflux would have been apparent. Ouabain-insensitive Na^+ efflux has been attributed to a transport mode of the $(Na^+ + K^+)$ATPase unaffected by ouabain (25) but, the ouabain insensitive ATPase activity rose by only 0.062 ± 0.008 mmol P_i /ℓcell·h in microwave exposed cells. To invoke the ouabain-insensitive Na^+ ATPase mode of the $(Na^+ + K^+)$ATPase as the explanation for the microwave effect on Na^+ efflux in the presence of ouabain one would be forced to accept the improbable transport stoicheometry of ~7 Na^+/1 ATP. Little, therefore, can be said concerning the effect of microwaves on the ouabain insensitive ATPase activity.

The observed increase in ouabain insensitive K^+ influx is assumed to be exclusively an increase in simple K^+ diffusion. There is only weak evidence of a facilitated, ouabain insensitive K^+ influx mechanism (117). Since passive cation permeation is

based more on particle charge and size, and less on elementary identity, one would expect a comparable increase in simple Na^+ diffusion to be part of the larger microwave effect on Na^+ efflux in the presence of ouabain. However, the results of the net Na^+ and K^+ flux experiments may indicate otherwise. Irradiated cells appear to accumulate Na^+ in the absence but not in the presence of ouabain while microwaves do not affect net K^+ movements in erythrocytes under either condition. The proposed increase in facilitated ouabain-insensitive Na^+/Na^+ exchange diffusion would have no effect on net Na^+ distribution. If simple Na^+ diffusion is unaffected by microwaves, the decrease in ouabain-sensitive Na^+ efflux could cause the net Na^+ accumulation observed in irradiated cells incubated with ouabain. In the presence of ouabain only the Na^+/Na^+ exchange-diffusion would be affected by microwaves and no change in net Na^+ distribution would occur. It follows that any Na^+ influx due to small changes in simple Na^+ diffusion may have been masked by the larger increase in ouabain-insensitive Na^+ efflux and was probably within the error limits of detection for net Na^+ distribution changes in the presence of ouabain.

The observation that within the same system microwaves can induce simultaneous but thermally opposing effects supports the hypothesis that microwaves, while capable of heating, can also exert more specific effects. The decrease in the $(Na^+ + K^+)$ATPase activity in irradiated erythrocytes cannot be attributed to microwave heating. A temperature increase would

have caused an increase in $(Na^+ + K^+)$ATPase activity. Thermal denaturation of protein can be ruled out since, beyond the effective temperature range no effect of microwaves was observed on any of the processes studied. That the reported effects of low-level microwaves on Na^+ and K^+ transport are all restricted to the same temperature range, 20 to 25 ^oC, indicate a common mechanism of interaction with microwaves as the physical basis for these effects.

The K^+-dependent ATPase activity, ouabain-sensitive Na^+ efflux and ouabain-sensitive K^+ influx are all a result of the activity of the $(Na^+ + K^+)$ATPase. A single interaction between microwaves and the $(Na^+ + K^+)$ATPase can be thus implicated as the cause of the observed changes in these processes. That microwaves may increase facilitated Na^+/Na^+ exchange-diffusion by a mechanism distinct from that mediated by the $(Na^+ + K^+)$ATPase (1) indicates an interaction between microwaves and yet another specific carrier. Microwaves also appear to act at a third site, possibly a permease, responsible for the effects observed on the simple K^+ (and probably Na^+) diffusion. Further, evidence exists that microwaves affect the activity of some other, K^+-independent ATPase(s) in the erythrocyte membrane. The affected processes share the common feature of being mediated by integral proteins of the erythrocyte membrane.

Although the function of integral membrane proteins is affected by the physical state of the lipid bilayer, microwave interaction with the bilayer is not probable. Despite a report

that concludes that microwaves may minutely influence membrane fluidity of simple lipid bilayer (102) the fluorescence of the fluidity-sensitive bilayer probe was unaffected within erythrocyte membranes exposed to 2450 MHz (3). Further, Na^+ leakage from both egg-PC and RBC-lipid vesicles was unaffected by microwaves. The mechanism whereby microwaves interact coherently with some vibration common to the different proteins or lipid/protein complexes that mediate the affected processes is thus most likely to ultimately explain the observed phenomena.

VIII REFERENCES

1 ALBERS, R.W. Biochemical aspects of active transport. Ann. Rev. Biochem. 36 (2): 727-756, 1967.

2 ALLIS, J.W., FROMME, M.L. Activity of membrane-bound enzymes exposed to sinusoidally modulated 2450 MHz microwave radiation. Radio Science 14(65):85-91, 1979.

3 ALLIS, J.W., SINHA, B.L. Fluorescence depolarization studies of red cell membrane fluidity. Biolectromagnetics 2:13-22, 1981.

4 BACH, S.A., LUZZIO, A.J., BROWNELL, A.S. Effects of radiofrequency energy on human gamma globulin. In:Biological Effects of Microwave Radiation-Symposium Proceedings Edited by M.F. Peyton. New York: Plenum Press, 1961, pp.117-134.

5 BANGAM, A.D. The liposome as a membrane model. In: Permeability and Function of Biological Membranes. Edited by L. Bolis, A. Katchalsky, R.D. Keynes, W.R. Loewenstein, and B.A. Pethica. Amsterdam: North-Holland Publishing Co. Amsterdam, 1970. pp. 195-206.

6 BARRON, C.I., BARAFF, A.A. Medical considerations of exposure to microwaves (radar). J. Amer. Med. Assoc. 168(9): 1194-1199, 1958.

7 BARANSKI, S., SZMIGIELSKI, S., MONITA, J. Effect of microwave irradiation in vitro on cell membrane permeability. In: Biological Effects and Health Hazards of Microwave Radiation. Edited by P. Czerski, K. Ostrowski, M.L. Shore, C.Silverman, M.J. Suess, and B. Waldeskog. Warsaw:Polish Medical Publishers, 1974. pp.173-177

8 BEAUGÉ, L., LEW, V.L. Passive fluxes of sodium and potassium across red cell membranes. In: Membrane Transport in Red Cells.Edited by J.C. Ellery and V.L. Lew. London: Academic Press Inc., 1977. pp.39-52.

9 BLACKMAN, C.F., BENANE, S.G., JOINES, W.T., HOLLIS, M.A., HOUSE, D.E. Calcium-ion efflux from brain tissue: power density versus internal field-intensity dependencies at 50 MHz RF radiation. Bioelectromagnetics 1:277-283, 1980.

10 BLACKMAN, C.F., ELDER, J.A., WEIL, C.M., BENANE, S.G., ELCHINGER, D.C., HOUSE, D.E. Induction calcium-ion efflux from brain tissue by radio frequency radiation: effects of modulation frequency and field strength. Radio Science 14(65):93-98, 1979.

11 BONTING, S. L., CARAVAGGIO, L. L. Studies on sodium-potassium-activated adenosinetriphosphatase IV. Correlation with cation transport sensitive to cardiac glycosides. Arch. of Bioch. and Biophys. 98: 413-419, 1962.

12 CARPENTER, R.L., LIVESTONE, E.M. Evidence for nonthermal effects of microwave radiation: Abnormal development of irradiated insect pupae. IEEE Trans. Microwave Theory Tech. MTT 19(2): 173-178, 1971.

13 CHRISTIE, R.V., LOOMIS, A.L. The relation of frequency to the physiological effects of ultra-high frequency currents. J. Exp. Med. 49: 303-321, 1929.

14 CLEARY, S.F. Biological effects of microwave and radio frequency radiation. C.R.C. Critical Reviews in Environmental Control 1:257-306, 1970.

15 CONNOR, W.P., CLARKE, R.P., SMYTH, C.P. The dielectric investigation of polypeptides. I. The dielectric increments of amino acid polypeptides. J. Amer. Chem. Soc. 64(1): 1379-1387, 1942.

16 CONNER, W.P., SMYTH, C.P. The dielectric investigation of polypeptides. II. The dispersion of simple amino acid polypeptides. J. Amer. Chem. Soc. 64:1870-1875, 1942.

17 DAILY, L.E. A clinical study of the results of exposure of laboratory personnel to radar and high frequency radio. U.S. Nav. Med. Bull. 41: 1052-1056, 1943.

18 D'ARSONVAL, J.A. Influence de la fréquence sur les effets physiologiques des courants alternatif. C.R. Acad.Sci. 116:630-633, 1893.

19 D'ARSONVAL, J. A., CHARRIN, A. Action de l'ectricité sur les toxines bacteriennes. French) S.R. Soc. Biol. 48: 121 -123, 1898.

20 DEVYATKOV, N.D. Influence of millimeter-band electromagnetic radiation on biological objects. Sov.Phys.-Usp. 16(4):568-569, 1974.

21 DODGE, C.H. Clinical and hygienic aspects of exposure to electromagnetic fields. In: Biological Effects and Health Implications of Microwave Radiation. Symposium Proceedings. Edited by S.F. Cleary. Washington, D.C.: (U.S. Public Health Service Publication BRH/DBF 70-2) U.S. Government Printing Office, 1970, pp.140-149.

22 DODGE, J.T., MITCHELL, C., HANAHAN, D.J. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch. Biochem. Biophys. 100:119-130, 1963.

23 DUMANSKIJ, J.D., SHANDALA, M.G. The biologic action and hygienic significance of electromagnitc fields of superhigh and ultrahigh frequencies in densely populated areas. In: Biological Effects and Health Hazards of Microwave Radiation. Edited by P. Czerski, K. Ostrowski, M.L. Suess, Ch. Silverman, M.L. Shore, B. Waldeskog. Warsaw: Polish Medical Publishers, 1974. pp.289-293.

24 DUNHAM, E.T., GLYNN, I.M. Adenosinetriphosphatase activity and the active movements of alkali metal ions. J. Physiol. (London) 156:274-293, 1961.

25 DUNN, M.J. The effects of transport inhibitors on sodium outflux and influx in red blood cells. J. Clin. Investigation 49 (10): 1804-1814, 1970.

26 ELFORD, B.C. Interaction between temperature and toxicity on cation transport in dog red cells. J. Physiol. (London) 246: 371-395, 1975.

27 ENOCH, H.G., STRITTMATTER, P. Formation and properties of 1000 A diameter single bilayer phospholipid vesicles. Proc. Nat. Acad. Sci. 76:145-149, 1979.

28 FAHIM, M.S., FAHIM, Z., DER, R. HALL, D.G., HARMAN, J. Heat in male contraception (hot water 60°C, infrared, microwave and ultrasound). Contraception 11:549-562, 1975.

29 FISHER, P.D., LAUBER, J.K., VOSS, W.A.G. The effect of low-level 2450 MHz CW microwave radiation and body temperature on the early embryonic development in chickens. Radio Science 14 (6S):159-163, 1979.

30 FISHER, P.D., VOSS, W.A.G., POZNANSKY, M.J. Transbilayer movement of $^{24}Na^{+}$ in sonicated phosphatidylcholine vesicles exposed to frequency-modulated microwave radiation. Bioelectromagnetics 2:217-225, 1981.

31 FISHER, P.D., POZNANSKY, M.J., VOSS, W.A.G. Effect of microwave radiation (2450 MHz) on the active and passive components of Na+ efflux from human erythrocytes. Radiation Research, in press, 1982.

32 FISKE, C.H., SUBBAROW, Y. The colorimetric determination of phosphorus. J. Biol. Chem. 66:375-400, 1925.

33 FOSTER, K.R., SCHEPPS, J.L., SCHWAN, H.P. Microwave dielectric absorption of muscle tissue: evidence for multiple absorption mechanisms between 1 and 18 GHz (abstract). Abstracts of the Bioelectromagnetics Symposium. Seattle, WA., June 18-22. Washington, D.C.: USNC-URSI, 1979, p373.

34 FRIEND, A.S., GARTNER, S.L., FOSTER, K.R., HOWE, H. The effects of high power microwave pulses on red blood cells and the relationship to transmembrane thermal gradients. IEEE Trans. Microwave Theory Tech. MTT 29 (12):1271-1276, 1981.

35 FRÖHLICH, H. The extraordinary dielectric properties of biological materials and the action of enzymes. Proc. Nat. Acad. Sci. U.S.A. 71(11): 4211-4215, 1975.

36 GARRAHAN, P.J., REGA, A.F. Cation loading of red blood cells. J. Physiol. (London) 193:459-466, 1967.

37 GLYNN, I.M. Sodium and potassium movements in human red cells. J. Physiol. (London) 134:278-310, 1936.

38 GLYNN, I.M. The action of cardiac glycosides on ion movements. Pharmacol. Rev. 16:381-407, 1964.

39 GOST Standard 12.1.006-76 Occupational safety standards systems. Electromagnetic fields of radiofrequency. General safety requirements (in Russian). Gosudarstvennyii Standard Soyuze USSR, 1976.

40 GRUNDLER, W., KEILMANN, F., FROHLICH, H. Resonant growth rate response of yeast cells irradiated by weak microwaves. Phys. Lett., A, 62(6):463-466, 1977.

41 GUIDOTTI, G. The composition of biological membranes. Arch. Int. Med. 129:194-201, 1974

42 GUNN,S.A., GOULD, T.C., ANDERSON, W.A.D. The effects of microwave radiation(24,000 mc) on the male endocrine system of the rat. In: Biological Effects of Microwave Radiation-Symposium Proceedings. Edited by M.F. Peyton. New York: Plenum Press, 1960, pp.99-116.

43 GUNN, R. B., TOSTESON, D. C. The effect of 2,4,6-trinitro-m-cresol on cation and anion transport in sheep red cells. J. General Physiol. 57:593-609, 1971.

44 HAMRICK, R.E., ZINKLE, J.G. Exposure of rabbit erythrocytes to microwave radiation. Radiat.Res. 63:164-168, 1975.

45 HANAHAN, D.J., BROCHERHOFF, H., BARRAN, E.J. The site of attack of phospholipase (lecithinase) A on lecithin: a re-evaluation. J. Biol. Chem. 235:1917-1923, 1960.

46 HEALER, J. Review of studies of people occupationally exposed to radiofrequency radiation. In: Biological Effects and Health Implications of Microwave Radiation. Symposium Proceedings. (U.S. Public Health Service Publication BRH/DBF 70-2) U.S. Government Printing Office: Washington, D.C., pp.90-97, 1970.

47 HESKETH, T.R., SMITH, G.A., HOUSLAY, M.D., McGILL, K.A., BIRDSALL, N.J.M., METCALFE, J.C., WARREN, G.B. Annular lipids determine the ATPase activity of a calcium transport protein complexed with dipalmitoyllecithin. Biochem. 15(19):4145-4151, 1976.

48 HEWLETT-PACKARD, Microwave Mismatch Error Analysis, Application note #56, Oct. 1967.

49 HIPPEL, A.R.von (Ed.) Dielectric Materials and Applications, Chapter 5, Tables of Dielectric Materials (Lab. for Insulation Res. MIT). Massachusetts: MIT Press, Cambridge, 1954, pp. 291-434.

50 HOFFMAN, J.F., KREGENOW, F.M. The characterization of new energy dependent cation transport processes in red blood cells. Ann. N.Y. Acad. Sci. 137:566-576, 1966.

51 HOKIN, L.E. MOKOTOFF, M., KUPCHAN, S.M. Alkylation of a brain transport adenosine triphosphate by the cardiac steroid site by strophanthidin-3-haloacetate. Proc. Nat. Acad. Sci. (U.S.) 55:794-804, 1966.

52 HUANG, C., THOMPSON, T.E. Preparation of homogeneous single-walled phosphatidylcholine vesicles. Methods Enzymol. 32:485-489, 1975.

53 ISMAILOV, E.S. On the mechanism of the effects of microwaves on the penetration in erythrocytes of the ions sodium and potassium. (In Russian) Nauchnye Doklody Vysshei Shkoly: Biologicheskie Nauki 3:58-60, 1971.

54 ISMAILOV, E.S. Changes of red cell permeability under the action of microwaves and their membranes. (Abstract) Fourth International Union for Pure and Applied Biophysics, Aug. 7-14, 1972. Moscow: Academy of Sciences of the USSR, 1972.

55 JAIN, M.K. The bilayer and related structures. In: Introduction to Biological Membranes. Edited by M.K. Jain and R.C. Wagner. New York: John Wiley and Sons, 1980. pp.54-87.

56 JAIN, M.K. Phase properties of bilayers. In: Introduction to Biological Membranes. Edited by M.K. Jain and R.C. Wagner. New York: John Wiley and Sons, 1980. pp. 88-117.

57 JAIN, M.K. Partitioning of solutes in bilayers: passive permeability. In: Introduction to Biological membranes. Edited by M.K. Jain and R.C. Wagner. New York: John Wiley and Sons, 1980. pp. 118-142.

58 JOINES, W.T., BLACKMAN, C.F. Power density, field intensity and carrier frequency determinants of RF-energy-induced calcium-ion efflux from brain tissue. Bioelectromagnetics 1:271-275, 1980.

59 JOYCE, C.R.B., WEATHERALL, M. Cardiac glycosides and the potassium exchange of human erythrocytes. J. Physiol. (London) 127:33P, 1955.

60 KAHLER, H., CHALKLEY, H.W., WOEGTLIN, C. The nature of the effect of a high frequency electric field upon Paramecium. Public Health Rep. 44(7):339-347, 1929.

61 KLIMKOVA-DUETSCHOVA, E. Neurologic findings in persons exposed to microwaves. In: Biological Effects and Health Hazards of Microwave Radiation. Edited by P. Czerski, K. Ostrowski, M.L. Shore, Ch. Silverman, M.J. Suess, B. Waldeskog, (eds). Warsaw: Polish Medical Publishers, 1974. pp.268-272.

62 KNUDSON, A., SCHAIBLE, P.J. Physiologic and biochemical changes resulting from exposure to an ultra-high frequency field. Arch. Pathol. 11:728-743, 1931.

63 LEVITT, D.G. The mechanism of the sodium pump. Biochem. Biophys. Acta. 604:321-345, 1980.

64 LIBURDY, R.P. Enhancement of protein elution during liquid gel chromatography by an externally applied RF electric field. In: The Mechanisms of Microwave Biological Effects. Edited by L.S. Taylor and A.V. Cheung. University of Maryland, 1979. p.18.

65 LILJENFORS, B., LOFGREN, H., Sodium ion diffusion through liposome membranes containing cerebroside. Chem. Phys. Lipids 29:111-120, 1980.

66 LINDAUER, G.A., LIU, L.M., SKEWES, G.W., ROSENBAUM, F.J. Further experiments seeking evidence of nonthermal biological effects of microwave radiation. IEEE Trans. Microwave Theory Tech. MTT 22:790-793, 1974.

67 LIU, M.L., CLEARY, S.F. Effects of microwave radiation on erythrocyte membranes. Radio Science 14 (6S):109-115, 1979.

68 LIU, L.M., ROSENBAUM, F.J., PICKARD, W.F. The relation of teratogenesis in Tenebrio molitor to the incidence of low-level microwaves. IEEE Trans. Microwave Theory Tech. MTT 23: 929-931, 1975.

69 LIVSHITS, N.N. The role of the nervous system in reactions to UHF electromagnetic fields. Biophys. 2(3):372-384, 1957.

70 LIVSHITS, N.N. The effect of ultra-high frequency field on the functions of the nervous system. Biophys. 3(4):409-421, 1958.

71 LORDS, J.L., DURNEY, C.H., BORG, A.M., TINNEY, C.E. Rate effects in isolated hearts induced by microwave irradiation. IEEE Trans. Microwave Theory Tech. MTT-21:834-836, 1973.

72 LORDS, J.L., MCARTHUR, G.R., DURNEY, C.H. Binding studies of receptor molecules under microwave irradiation. (Abstract) Bioelectromagnetics 1(2):206, 1980.

73 LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., RANDALL, R.J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275, 1951.

74 LUBOWITZ, H., WHITTAM, R. Ion movements in human erythrocytes independent of the sodium pump. J. Physiol. (London) 202:111-131, 1969.

75 MARCHESI, V.T., FURTHMAYER, H., TOMITA, M. The red cell membrane. Ann. Rev. Biochem. 45:667-698, 1976.

76 MARCY, H.O., WYMAN, J. Anomalous dispersion of dipolar ions. J. Amer. Chem. Soc., 63:3388-3397, 1941.

77 McAFFEE, R.D. The neural and hormonal response to microwave stimulation of peripheral nerves. In:Biological Effects and Health Implications of Microwave Radiation. (US Public Health Service Publication BRH/DBF 70-2) US Government Printing Office: Washington D.C., pp. 150-153, 1970.

78 McAFFEE, R.D., BERGER, C., PIZZOLATO, P. Neurological effect of 3cm microwave irradiation. In: Biological Effects of Microwave Radiation-Symposium Proceedings. Edited by M.F. Peyton. New York: Plenum Press, 1961. pp.251-260.

79 MCREE, D.I., WACHTEL, H. Elimination of microwave effects on the vitality of nerves after active transport has been blocked. (Abstract) URSI:National Radioscience meeting, Bioelectromagnetics Symposium June 18-22, 1979. Program and Abstracts, Seattle, WA. 1979.

80 MELLON, R.R., SZYMANOWSKI, W.T. An effect of short electric waves on diphtheria toxin independent of the heat factor. Science 72(1859):174-175, 1930.

81 OLCERST, R.B., BELMAN, S., EISENBUD, M., MUMFORD, W.W., RABINOWITZ, J.R. The increased passive efflux of sodium and rubidium from rabbit erythrocytes by microwave radiation. Radiat. Res. 82:244-256, 1980.

82 OLSEN R.G., LORDS, J.L., DURNEY, C.H. Microwave-Induced chronotropic effects in the isolated rat heart. Ann. Biomed. Eng. 5:395-409, 1977.

83 PAPAHADJOPOULOS, D., NIR, S., OHKI, S. Permeability properties of phospholipid membranes. Effect of cholesterol and temperature. Biochim. Biophys. Acta. 266:561-583, 1971.

84 PAPAHADJOPOULOS, D., JACOBSON, K., NIR, S., ISAC, T. Phase transitions in phospholipid vesicles: Fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. Biochim. Biophys. Acta. 311:330-348, 1973.

85 PAPAHADJOPOULOS, D. Phospholipids as model membranes: Monolayers, bilayers and vesicles. In: Form and Function of Phospholipids (BBA) Library, Vol. 3). Edited by G.B. Ansell, J.N. Hawthorne and R.M.C. Dawson. Amsterdam: Elsevier Scientific Publishing Co., 1973. pp.141-169.

86 PETERSON, D. J., PARTLOW, L. M., GANDHI, O.P. An investigation of the thermal and a thermal effects of microwave irradiation on erythrocytes. IEEE Trans. Biomed. Eng. BME 26:428-436, 1979.

87 PEYTON, M.F. (ed) Biological Effects of Microwave Radiation-Symposium Proceedings. New York: Plenum Press, 333 pg. 1961

88 POST, R.L., MERRITT, C.R., KINSOLVING, C.R., ALBRIGHT, C.D. Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in human erythrocyte. J. Biol. Chem. 235(6):1796-1802, 1960.

89 POST, R.L. A perspective on sodium and potassium ion transport adenosine triphosphatase. In: Cation Flux across Biomembranes. Edited by Y. Mukohata and L. Packer. New York: Academic Press, 1979. pp.3-20.

90 QUINN, P.J., CHAPMAN, D. The dynamics of membrane structure. CRC Crit. Rev. Bioch. 8:1-117, 1980.

91 ROBINSON, N., NIEVEL, J.G., ANDERSON, J. Histochemical and ultrastructural analysis of the effect of electrmomagnetic radiation on rat liver and brain. (Abstract) Biochem. Soc. Trans. 2:1359-1360, 1974.

92 ROBINSON, J.D., FLASHNER, M.S. The (Na^+ + K^+)-activated ATPase: Enzymatic and Transport properties. Biochim. Biophys. Acta S49:145-176, 1979.

93 SAAD, T.S. (Editor-in-Chief). Microwave Engineers Technical and Buyers Guide Edition of The International Microwave Journal. Dedham, Mass.: Horizon House - Microwave, Feb. 1, 1968.

94 SADCIKOVA, M.N. Clinical manifestations of reactions to microwave irradiation in various occupational groups. In: Biological Effects and Health Hazards of Microwave Radiation.Edited by P. Czerski, K. Ostrowski, M.L.Shore, Ch. Silverman, M.J. Suess, B. Waldeskog. Warsaw: Polish Medical Publishers, 1974. pp.261-267.

95 SANDERS, A.P., SCHAEFER, D.J., JOINES, W.T. Microwave effects on energy metabolism of rat brain. Biolectromagnetics 1:171-181, 1980.

96 SCHERESCHEWSKY, J.W. The physiological effects of currents of very high frequency (134,000,000 to 8,300,000 cycles per second). Public Health Rep. 41:927-939, 1926.

97 SCHULTZ, S.G. Ion-coupled transport across biological membranes. In: Membrane Physiology. Edited by T.E. Andreoli, J.F. Hoffman and D.D. Fanestil. New York: Plenum Publishing Corp., 1980. pp. 273-286.

98 SCHUURMANS-STEKHOVEN, F., BONTING, S.L. Transport Adenosine triphosphatases: Properties and functions. Physiol. Rev. 61:1-76, 1981.

99 SCHWAN, H.P. Field interaction with biological matter. Ann. N.Y. Acad. Sci.303:198-213, 1977.

100 SCHWOCH, G., PASSOW, H. Preparation and properties of human erythrocyte ghosts. Mol.and Cell. Biochem. 2(2):197-218, 1973.

101 SIEKIERZYNSKI, M. A study of the health status of microwave workers. In: Biological Effects and Health Hazards of Microwave Radiation. Edited by P. Czerski, K. Ostrowski, M.L. Shore, Ch. Silverman, M.J. Suess, B. Waldeskog. Warsaw: Polish Medical Publishers, 1974. pp273-280.

102 SHERIDAN, S.P., GABER, B.P., CAVATROTA, F. SCHOEN, P.E. Molecular level effects of microwaves on natural and model membranes: A raman spectroscopic investigation. (Abstract) URSI:National Radioscience meeting, Bioelectromagnetics Symposium June 18-22, 1979. Program and Abstracts, Seattle, WA. pp.468, 1979.

103 SKOU, J.C. Enzymatic basis for active transport of Na+ and K^+ across cell membrane. Physiol. Rev. 45:596-617,1965.

104 SKOU, J. C., HILBERG, C. The effect of sulfhydryl-blocking reagents and of urea on the (Na+ + K+)-activated enzyme system. Biochim. Biophys. Acta. 110:359-369, 1965.

105 SOKAL, R.R., ROHLF, F.J. BIOMETRY: The Priciples and Practice of Statistics in Biological Research. San Francisco: W.H. Freeman and Co., 1969.

106 SOLOMON, A.K. The permeability of the human erythrocyte to sodium and potassium. J. Gen. Physiol. 36(1)57-110, 1952.

107 STRAUB, K.D., CARTER, P. Effects of electromagnetic fields on microsomal ATPase and mitochondrial oxidative phosphorylation. Ann. N. Y. Acad. Sci. 247:292-300,1975.

108 SUSSKIND, C. The "Story" of nonionizing radiation research. Bull. N. Y. Acad. Med. 55(11):1152-1163, 1979.

109 SZYMANOWSKI, W.T., HICKS, R.A., The biological action of ultra-high frequency currents. J. Infectious Diseases 50(1):1-25, 1935.

110 TAYLOR, L.S. The mechanisms of athermal microwave biological effects. Bioelectromagnetics 2:250-267,1981.

111 TINGA, W.R., NELSON, S.O. Dielectric Properties of Materials for Microwave Processing - Tabulated. J. Microwave Power 8 (1):23-65, 1973.

112 TINNEY, C.E., LORDS, J.L., DURNEY, C.H. Rate effects in isolated turtle hearts induced by microwave irradiation. IEEE Trans. Microwave Theory Techn. MTT-24:18-24,Jan. 1976.

113 UMMERSEN, C. van The effect of 2450 mc radiation on the development of the chick embryo. In: Biological Effects of Microwave Radiation-Symposium Proceedings. Edited by M.F. Peyton. New York: Plenum Press, 1961. pp. 201-220 .

114 WARREN, G.B., HOUSLAY, M.D., METCALFE, V.C., BIRDSALL, N.J.M. Cholesterol is excluded from the phospholipid annulus surrounding an active calcium transport protein. Nature 255:684-687, 1975.

115 WEBB, S.J., BOOTH, A.D., Absorption of microwaves by microorganisms. Nature 222:1199-1200, 1969.

116 WEED, R., EBER, J., ROTHSTEIN, A. Interaction of Mercury with human erythrocytes. J. Gen. Physiol. 45:395-410, 1962.

117 WHITTAM, R., WHEELER, K.P. Transport across cell membranes. Ann. Rev. Physiol. 32:21-60, 1970.

118 YEARGERS, E.K., LANGLEY, J.B., SHEPPARD, A.P., HUDDLESTON, G.K. Effects of microwaves radiation on enzymes. Annals New York Acad. of Sci. 247:301-304, 1976.

119 YOUNG, J.D., ELLORY, J.C. Red cell amino acid transport. In: Membrane Transport in Red Cells. Edited by J.C. Ellory and V.L. Lew. Academic Press Inc, (London), 1977. pp.301-325.

120 ZOELEN, E.J.J. van, DIJCK, P.W.M. van. KRUIJFF, B. de, VERKLEIJ, A.J., DEENEN, L.L.M. van Effect of glycophorin incorporation on the physico-chemical properties of phopholipid bilayers. Bioch. Biophys. Acta. 514:9-24, 1978.

Additional References

121 GRANT, E.H., SHEPPARD, R.J., SOUTH, G.P. Dielectric Behavior of Biological Molecules in Solution. Clarendon Press, (Oxford), 1978, 237pp.

122 BAWIN, S.M., ADEY, W.R. Sensitivity of calcium binding in cerebral tissue to weak environmental electric fields oscillating at low frequency. Proc. Nat. Acad. Sci. (USA). 73:1999-2003, 1976.